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SECTION MEETINGS

OHIO VALLEY

Children's Hospital, Cincinnati  
State Board of Health, Indianapolis

October 23, 1948  
January 29, 1949

PACIFIC COAST

Stanford University

April 9, 1949

SOUTHERN

Tulane University

April 8, 1949

SOUTHERN CALIFORNIA

University of California, Los Angeles

April 14, 1949

WESTERN NEW YORK

University of Rochester

February 19, 1949

17058. A Comparative Study of the Local Toxic Action of Mercurial Diuretics.

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The mercurial diuretics have today a secure place in the management of the edematous patient because of their effectiveness and dependability. However they give rise, on occasion, to various side effects of which irritation at the site of injection and an acute toxic action on the heart are the most significant. This latter action may sometimes be fatal<sup>1-3</sup> and in an attempt to eliminate it a new type of mercurial has recently been made available under the name Thiomerin.\*

This drug is identical with Mercuzanthin (Mercurophylline, USP XIII) except that it contains sodium mercapto acetate in chemical combination with the mercury instead of theophylline. In animal experiments it failed to produce the typical ventricular tachycardia and fibrillation which occurs after the other diuretics.<sup>4</sup> Thiomerin also appears to be less toxic to tissue and has been proposed for clinical use by subcutaneous injection. It therefore seemed desirable to make a detailed comparison of the mercurial diuretics with respect to the reaction which they produce at the site of injection. Thiomerin, Mercuzanthin, and Mercuhydrin were used in this study.

*Subcutaneous Injection in Mice.* It has

<sup>1</sup> DeGraff, A. C., and Nadler, J. E., *J. Am. Med. Assn.*, 1942, **119**, 1006.

<sup>2</sup> Ben-Asher, Solomon. *Ann. Int. Med.*, 1946, **25**, 711.

<sup>3</sup> Volini, Italo, Levitt, R. O., and Martin, Richard, *J. Am. Med. Assn.*, 1945, **128**, 12.

\* Thiomerin has also been identified as MT6.

<sup>4</sup> Lehman, R. A., *Proc. Soc. Exp. Biol. and Med.*, 1947, **64**, 428.

previously been shown in rabbits, that addition of theophylline to a parent mercurial such as Salyrgan or Mercuzan, protects the skin from the severe necrosis following intradermal injection.<sup>5</sup> However, this technic was not found to give a sufficiently sensitive or consistent response for the study of mercurials of a less irritant character. The albino mouse was selected as a more suitable animal since, unlike the rabbit, the skin over the abdomen is thin enough so that necrosis cannot escape detection, and large enough groups of animals can be used to evaluate the effect of individual variation.

Three groups of 5 mice in the weight range of 20 to 30 g were selected at random and the hair carefully clipped over the abdomen. The first group received Thiomerin, the second Mercuzanthin and the third Mercuhydrin all by subcutaneous injection in a dose of 0.03 cc of undiluted solution as supplied for clinical use (40 mg of mercury per cc). A one quarter cc tuberculin syringe with a 27 gauge hypodermic needle was used with the bevel up. All the animals were injected on the same day. Approximately 24 hours after the injection the mice were examined for evidences of toxicity. They were then anesthetized with a barbiturate and photographed in color. These photographs appear as Fig. 1.

It will be observed that in the case of Mercuzanthin the destruction of tissue has progressed in all animals to the point of skin breakdown over a substantial area of the abdomen. The very dark discoloration accompanying some of the lesions suggests the presence of extravasated blood. In every case there is a white ring surrounding the area of necrosis which is presumably due to inflammatory exudate.

The response to Mercuhydrin is of similar character but somewhat less severe, more superficial, and involves a smaller area of skin. Nevertheless perforation of the skin will be observed in the first animal on the left in Fig. 1. Extravasation of blood appears not to have occurred in this group.

None of the animals injected with Thiomerin shows pathology which can be detected on gross examination.

*Intramuscular Injection in Rats.* Because of these marked differences in local toxicity, studies were undertaken to compare the histopathology occurring after injection of Thiomerin, Mercuzanthin and Mercuhydrin. For this purpose the tibialis anterior muscle of the rat was selected as a more homogeneous tissue than the skin for the precise administration of a measured volume of drug. Each rat was etherized very lightly, the hair shaved from the leg and 0.03 cc of undiluted drug was injected into the belly of the muscle using a one-quarter cc syringe and 27 gauge needle. Twenty of the rats received saline control injections into the corresponding muscle of the opposite leg. The rats were killed by a blow on the head after a period of 4, 24, or 96 hours. The skin was stripped away and the injected muscle gently freed from adherent muscle bundles and dissected free. Muscles were fixed in Bouin's solution, embedded in paraffin and cut at 10  $\mu$ . Each muscle was sectioned serially at intervals of 500  $\mu$ . The sections were examined by the pathologist without foreknowledge of the treatments given and the pathology was scored as "none", "slight", "moderate", "marked", or "very marked" in the following categories: "disruption of muscle integrity", "cellular infiltration", and "edema". The results are given in the Table.

*Disruption of Muscle Integrity.* It appears from the Table that after 4 or 24 hours the 3 drugs show definite disruption of tissue in distinction to the saline control. After 96 hours the disruption is less than at 4 or 24 hours in the case of Mercuzanthin and Mercuhydrin while Thiomerin injected muscles now show no disruption and cannot be distinguished from the controls.

*Cellular Infiltration.* After 4 hours all of the drugs as well as saline have produced a slight cellular response as indicated in the Table. Twenty-four hours after injection the response to saline has not increased while that to the three mercurials has increased a great deal. After 96 hours the infiltration has disappeared in the case of Thiomerin and

<sup>5</sup> DeGraff, A. C., Batterman, R. C., and Lehman, R. A., *Proc. Soc. Exp. Biol. and Med.*, 1938, **38**, 373.

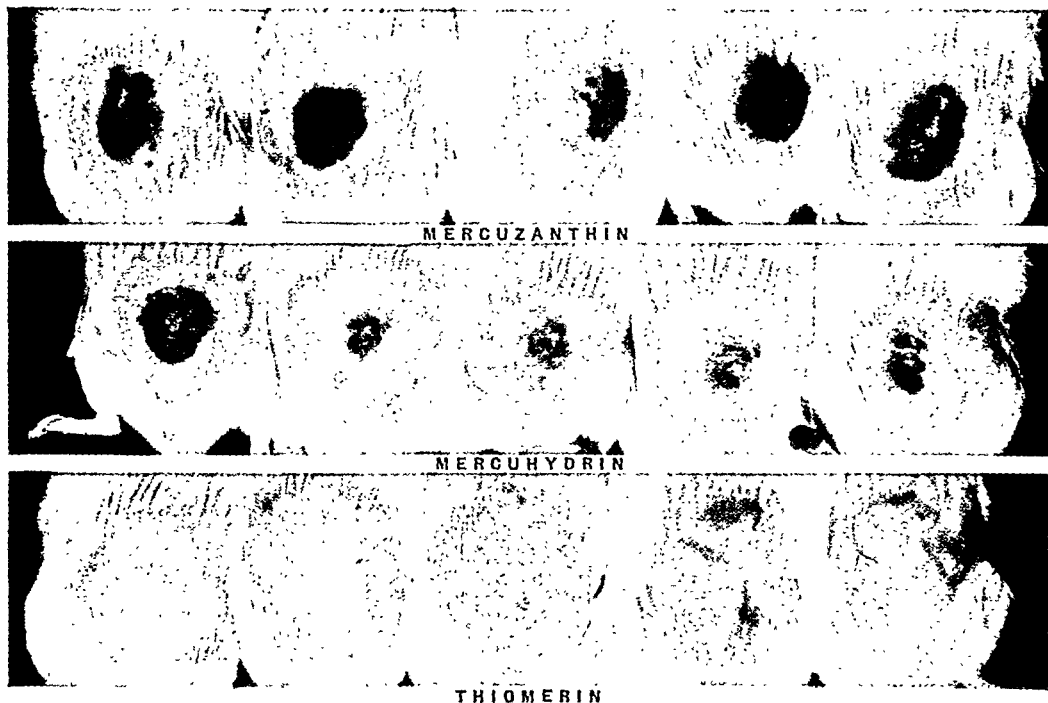


FIG. 1

Photographs of mice 24 hours after the subcutaneous injection of 0.03 cc. of a mercurial diuretic at a concentration of approximately 40 mgm of mercury per cc. All of the mice in a row received the same treatment. *Top Row:* Mercuzanthin (Mercurophylline USP XIII); *Middle Row:* Mercuhydrin (Meralluride); *Bottom Row:* Thiomerin.



TABLE I  
 Scores for Examination of Muscle Sections After Injection of Mercurial Diuretics.\*

Drug	4 hr			24 hr			96 hr		
	DI	CI	ED	DI	CI	ED	DI	CI	ED
Saline	0	+	0	0	0	0	0	0	0
	0	+	+	0	+	0	0	0	0
	0	+	+	0	+	0	0	0	0
	0	+	+	0	+	0	0	+	0
	+		+	0	+	0	++	+++	+
				0	+	+			
				0	+	+			
				0	+	+			
				+	+	+			
				+	++	+			
Thiomerin	+	+	+	+	++	+	0	0	0
	+	+	++	+	++	++	0	0	0
	+	+	++	++	++	++	0	+	0
	++	++	++	++	++	++	0	+	0
	++	++	++	++	++	++	0	+	+
				++	+++	++			
				++	+++	++			
				++	+++	++			
				++	+++	++			
				++	+++	+++			
Mercuzanthin	+	+	+	++	++	+	0	++	+
	+	+	+	++	++	++	+	++	+
	++	+	+	+++	+++	++	++	++	+
	++	+	++	+++	+++	++	++	++	++
				+++	+++	+++	++	+++	++
Mercuhydrin	+	+	+	+	++	+	+	++	+
	++	+	+	++	++	++	++	+++	+
	++	+	++	++	++	++	++	+++	+
	+++	+	++	+++	+++	++	++	+++	+
	+++		++	+++	+++	++	++	+++	+

\* 0 none; + slight; ++ moderate; +++ marked; ++++ very marked.

DI, disruption of muscle integrity; CI, cellular infiltration; ED, edema.

saline but is still present in the case of Mercuzanthin and Mercurhydrin.

**Edema.** Four hours after injection edema is present in every instance although saline shows less than the 3 drugs. The edema persists for at least 24 hours. After 96 hours it has disappeared from the saline and Thiomerin injected muscles but is still present in the others. It should be pointed out in this connection that absorption of these drugs is substantially complete one hour after injection.<sup>6</sup>

The character of the response is illustrated in Fig. 2, 3, and 4. Fig. 2 is typical of the response 24 hours after injection of any one of the 3 mercurials and is characterized by the infiltration of polymorphs, lymphocytes and the appearance of edema fluid. The response to saline was similar but quantitatively less extensive. Fig. 3 illustrates the appearance

of muscle 96 hours after injection of Mercuzanthin or Mercurhydrin and shows not only the persistence of the inflammatory exudate suggesting continued irritation but also the development of a reparative response as manifested by newly-formed connective tissue. Fig. 4 shows essentially normal muscle which was consistently observed 96 hours after injection of Thiomerin or saline.

**Discussion.** It has long been taken for granted that mercury compounds in which at least one valence bond bears an ionizable group are highly irritant to tissues. Thus Salyrgan with the configuration  $\text{RHg}-\text{OH}$  is severely and rapidly necrotizing.<sup>5</sup> The introduction of Mercuzanthin provided a compound of structure  $\text{RHg}-\text{N}<$  in which the irritant action of the mercury has been considerably reduced by combination with theo-

## LOCAL TOXIC ACTION OF MERCURIAL DIURETICS

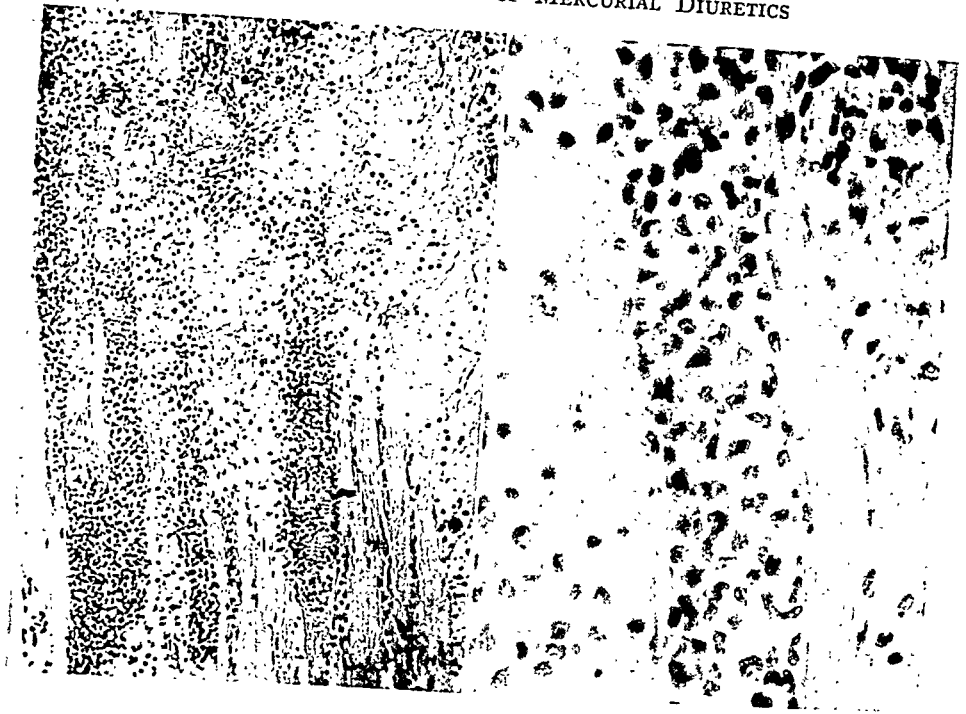


FIG. 2.

Section of rat muscle illustrating the response to Mercuzanthin, Mercuhydrin or Thiomerin 24 hr after inj. Left— $\times 100$ ; right— $\times 450$ .



FIG. 3.

Section of rat muscle illustrating the response to Mercuzanthin or Mercuhydrin 96 hr after inj. Left— $\times 100$ ; right— $\times 450$ .



FIG. 4.

Section of rat muscle 96 hr after inj. of Thiomerin or saline showing essentially normal tissue.  $\times 100$ .

phylline. Nitrogen compounds of similar structure (such as succinimide) can be substituted for theophylline with comparable effect.<sup>6,7</sup> Salyrgan-theophylline and Mercuhydrin are theophylline-bearing drugs of this class. In Thiomerin the second valence bond of the mercury has been bound as a mercaptide to give a substance of the formula  $\text{RHg-S-}$  and this modification has been shown above to result in a drug of still less toxicity. In a previous report it was indicated that the addition of a sulfhydryl compound to a conventional diuretic will cause precipitation of the theophylline indicating that the  $\text{Hg-S}$  bond is chemically more stable than the  $\text{Hg-N}$ .<sup>4</sup> Hence the results here reported would be consistent with the conclusion that local irritant action in the mercurial diuretic series is proportional to the reactivity of the mercury which they contain. A logical extension of this series would be the compound  $\text{RHg-C} \leq$  which might be expected to show even less local irritant action than Thiomerin. However, such a mercurial would also be expected not to have therapeutic activity.

It is beyond the scope of this report to discuss the effect of the  $\text{Hg-S}$  bond on diuretic

activity. Clinical studies<sup>8-10</sup> seem to indicate that Thiomerin is at least as effective as the other mercurials in this respect.

It may be mentioned in passing that the sensitivity of mice to the subcutaneous injection of mercurials was found to be greater in the summer than in the winter months. All of the injections for the experiment which is illustrated in Fig. 1 were accordingly given on the same day (September) so that the comparison would be valid. In this connection the work of DiPalma and coworkers<sup>11</sup> on seasonal variations in the sensitivity of the skin to the production of reactive hyperemia is of interest.

**Summary and Conclusions.** Three mercurial diuretics, Thiomerin, Mercuzanthin and Mercuhydrin have been compared with respect to the tissue reaction which they produce. Thiomerin was tolerated by mice on subcutaneous injection without exhibiting gross pathology while Mercuzanthin and Mercuhydrin gave

<sup>6</sup> Lehman, R. A., and Dater, Arnold, *J. Pharm. Exp. Therap.*, 1938, **63**, 443.

<sup>7</sup> Hunt, W. H., Walter, L. A., and Fosbinder, R. J., *J. Am. Pharm. Assn., Sci. ed.*, 1942, **31**, 278.

<sup>8</sup> Herrmann, George R., Chriss, John W., Hejmanek, Milton R., and Sims, Paul M., *Texas State J. Med.*, 1949, **45**, 79.

<sup>9</sup> Grossman, J., Weston, R. E., Edelman, I. S., and Leiter, L., in press.

<sup>10</sup> Batterman, Robert C., Unterman, David, and DeGraff, Arthur C., *J. Am. Med. Assn.*, in press.

<sup>11</sup> DiPalma, J. R., Reynolds, S. M. R., and Foster, F. I., *Am. Heart J.*, 1942, **23**, 377.



rise to necrosis of the skin under the same conditions. After intramuscular injection in rats all three drugs gave an early inflammatory response characterized by the appearance of a polymorphonuclear exudate. In the case of Thiomerin this exudate was entirely resorbed without evidence of residual damage. After injection of Mercuzanthin, or Mercuhydrin, however, the irreversible nature of the re-

sponse was indicated by marked fibroblastic proliferation.

These results would seem to furnish an adequate experimental basis for the clinical use of Thiomerin by subcutaneous injection.

The authors wish to thank the Misses Florence Katine and Josephine Brosseau for able technical assistance.

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### 17059. Observations of Nitrite-Induced Postural Syncope in Patients with Mental Disease.

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Earlier work<sup>1</sup> discussed the vasomotor system in schizophrenia; it was concluded that no structural vascular defect exists and that schizophrenic patients often exhibit acral vasoconstriction. Investigation of the reactivity of postural vasoconstrictor mechanisms has also been made. Nitrite dilates vessels by relaxing smooth muscle, but does not prevent the action of nervous impulses on this muscle; accordingly, the degree to which the vasoconstriction overcomes the effect of nitrite affords data on the reactivity of postural vasoconstrictor mechanisms.

**Material and methods.** Twenty-four patients were studied. Their diagnoses varied (Table I). Four (Table II), all schizophrenics were studied only after lobotomy. Twenty-four normal subjects were also observed. After reassurance the subjects came to the laboratory fasting or 3 or 4 hours after a last meal. Patients were not permitted to smoke before or during experiments. Each subject was given 3 grains of sodium nitrite by mouth and allowed to rest for 30 minutes. He then lay on a board which had no protuberances except for a bicycle seat at right angles to it; this the patient straddled. After control observations were made, the board was tilted 60 degrees leaving the patient head-up with legs hang-

ing. Systolic blood pressure was measured every few minutes by palpation. Auscultatory measurements were not made since they may be markedly in error with changing vasomotor tone; an arterial cannula was considered undesirable for measuring arterial pressure in this study. If syncope did not occur within forty minutes after tilting the patient, the observations were ended and were repeated no sooner than 2 days later, using 7 grains of sodium nitrite.

**Observations.** Subjects were classified as follows:

Group I. Syncope during tilting after 3 grains of sodium nitrite, *i.e.*, no increase in vasomotor reactivity (Fig. 1).

Group II. Syncope during tilting after 7 grains but not after 3 grains of sodium nitrite, *i.e.*, possible increase in vasomotor reactivity (Fig. 2).

Group III. Fall in blood pressure during tilting but no syncope after 7 grains of sodium nitrite, *i.e.*, definite increase in vasomotor reactivity (Fig. 3).

Group IV. Rise in blood pressure during tilting after 7 grains of sodium nitrite, *i.e.*, marked increase in vasomotor reactivity (Fig. 4).

Twenty-two normal subjects were in Group I and 2 in Group II. Seven of the 11 non-lobotomized schizophrenic patients exhibited

<sup>1</sup> Altschule, M. D., and Sulzbach, W. M., *Arch. Neurol. and Psychiat.*, 1949, 61, 44.

TABLE I.  
Vasomotor Response to Tilting After Administration of Nitrite.

Diagnosis	No. of cases				
	Groups I	II	III	IV	Total
Normal	22	2	—	—	24
Schizophrenia	3	1	5	2	11
Organic disease—post asphyxial	—	—	1	—	1
Involuntional psychosis, paranoia	1	—	—	—	1
Manic-depressive, manic	—	1	—	—	1
Anxiety neurosis	—	—	1	—	1
Psychoneurosis, reactive depression	1	—	1	—	2
Chronic alcoholism	—	2	1	—	3
Total, non-schizophrenic	2	3	4	0	9

TABLE II.  
Vasomotor Response to Tilting After Administration of Nitrite in Lobotomized Patients.

Case	Group		Diagnosis
	Before	After	
We	III	I	Schizophrenia, paranoid
Wi	II	III	" "
B	—	I	" catatonic
S	—	III	" "
L	—	IV	" "
Wo	—	IV	Schizophrenia, other types
Z	I	I	" " " "

a definite or marked increase in postural vasomotor reactivity. Of the 9 patients, with diagnoses other than schizophrenia, 4 exhibited a

definite increase. Of the 7 schizophrenic patients studied at least 4 months after lobotomy, 4 showed definite or marked increases (Table II). No consistent change in vasomotor reactivity developed after operation.

The schizophrenic patient showed no correlation between the presence of acral cyanosis under control conditions and increase of vasomotor reactivity; however, acral cyanosis developed, or if present, became intensified when the patients were tilted to the head-up position.

Mood or thought content changed sharply in some instances. A patient with involuntional psychosis was studied 11 times; each time

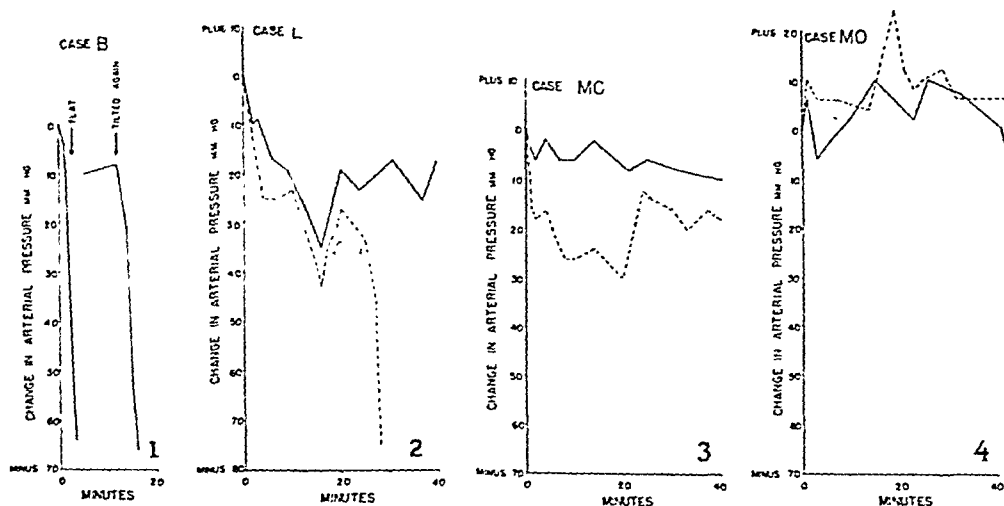


FIG. 1. Group I Reaction. Syncope after 3 grains of sodium nitrite.

FIG. 2. Group II Reaction. Syncope after 7 grains (dotted line) but not after 3 grains of sodium nitrite (solid line).

FIG. 3. Group III Reaction. Fall in blood pressure but no syncope after 3 grains (solid line) or 7 grains (dotted line) of sodium nitrite.

FIG. 4. Group IV Reaction. Rise in blood pressure after 3 grains (solid line) and after 7 grains (dotted line) of sodium nitrite.

distressing hallucinations of violent events developed a few minutes after she had been tilted up and when decreases in blood pressure were only slight. They persisted until she was returned to the flat position, when they disappeared, leaving the patient shaken but cognizant that the visions had been hallucinations. A patient with psychosis due to asphyxial cerebral damage became fearful when her blood pressure began to fall after she was tilted up. She spoke spontaneously for the first time of the fire during which she had almost died and which marked the onset of her illness. She described how her son had died and she also brought out other material previously suppressed. A manic-depressive patient in the manic phase became more active when her blood pressure began to fall after she was tilted up; she showed excitement and rambling speech alternating with moodiness and weeping. The patients with reactive depressions, psychoneurosis and chronic alcoholism showed no change in mood or mental content. The 15 schizophrenic patients observed showed various psychological reactions. In 3 instances syncope occurred so quickly that no evaluation of changes in thinking could be made. Two young patients with recent onset of schizophrenia became more self-absorbed and showed expressions suggestive of hallucinatory mental content when their blood pressures were falling. The remaining patients, all examples of schizophrenia of long duration, showed no definite psychological change when tilted in the head-up position although there was some decrease in blood pressure in half of them.

*Discussion.* The data indicate that increased reactivity of the vasomotor mechanisms involved in postural changes may exist in patients with mental and emotional disorders. This finding is not limited to schizophrenia. The mechanism of the increased vasomotor reactivity observed here is not evident. The vasomotor phenomena here observed are of interest in that they indicate the occurrence of disturbances of functions other than those concerned with thought, mood or perception in mental disease. The similarity of findings in schizophrenia and in

non-schizophrenic disorders does not support the concept that the former is accompanied by a specific change in autonomic function. Moreover, the presence of increased reactivity of the postural vasomotor mechanisms in schizophrenia, negates the belief that the autonomic nervous system is less responsive than normal in this disease. The common finding of increased reactivity of vasomotor mechanisms in schizophrenic patients here and as previously discussed<sup>1</sup> suggests that the conclusion that such increased vasomotor reactivity is the precursor of hypertension is erroneous since the incidence of the latter is low in schizophrenia. The patients who developed activation of their psychoses when placed in the head-up position are of interest. These changes began and often became marked at a time when changes in the general circulation were too small to be noteworthy; nevertheless, at this time significant changes in cerebral circulation already must have been in progress.<sup>2</sup> The events in these patients suggest the wisdom of caution in ascribing the onset of, or changes in overt psychotic manifestations definitely to psychological factors whenever evidences of changes in physiological status of the brain are absent on superficial examination.

Studies made here on lobotomy, though few, indicate that it causes no distinctive change in postural vasomotor reactivity if time for complete healing is allowed. Rinkel *et al.*<sup>3</sup> found increases in the response to injected epinephrin in patients after lobotomy. These findings may possibly indicate over-reactivity of the sympathetic nervous system, or, on the other hand, may merely indicate an altered pharmacological response to a substance given greatly in excess of physiological quantities. At any rate, the fact that Rinkel *et al.*<sup>3</sup> made their studies a few weeks after lobotomy, suggests that the changes they found are manifestations of an irritative phenomenon and are not due to division of the tracts cut during the operation.

<sup>2</sup> Lennox, W. G., Gibbs, F. A., and Gibbs, E. L., *Arch. Neurol. and Psychiat.*, 1935, **34**, 1001.

<sup>3</sup> Rinkel, M., Greenblatt, M., Coon, G. P., and Solomon, H. C., *Arch. Neurol. and Psychiat.*, 1947, **58**, 570.

*Summary and conclusions.* In patients with mental or emotional disorders, increased activity of vasomotor mechanisms responsive to postural change may occur; this is observed irrespective of diagnosis. In some psychotic patients disturbances in mental content and mood, consisting of intensification of pre-existing psychotic manifestations, occurred at times

when it may be assumed that cerebral blood flow was diminished. Observations suggest that lobotomy causes no distinctive change in postural vasomotor reactivity when time for complete healing is allowed.

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## 17060. Effect of Autolyzed Yeast, Yeast Nucleic Acid and Related Substances on Body-Temperatures of Rats.\*

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It is common practice to produce fever experimentally in rats by means of the subcutaneous injection of a solution of autolyzed yeast. During the routine use of this procedure, we found that administration of the autolyzed yeast intraperitoneally produced a fall instead of a rise in body-temperature. In the present paper we are reporting this phenomenon and some efforts to determine what constituent or constituents of the yeast may be responsible for the temperature effects. The activities of autolyzed yeast, yeast nucleic acid, and the nucleic acid derivatives, guanine, uracil, xanthine, and allantoin were investigated.

*Materials.* The autolyzed yeast was prepared in the usual way by making a suspension of 15 g of commercial baker's yeast in 100 cc of water. This suspension was allowed to stand for several days at 37°C with occasional shaking. The solid material was then separated in the centrifuge and the clear, dark brown, supernatant "autolysate" was used as the experimental material. The magnesium salt of yeast nucleic acid was prepared according to the method of Baumann.<sup>1</sup> All nucleic acid derivatives used were Eastman products and when necessary were brought to

essentially neutral condition before administration.

*Experimental.* The experimental animals were young adult white rats about equally divided between males and females in each experiment. The animals were fasted overnight before use. High colonic temperatures were taken with mercury thermometers by means of the technic previously described.<sup>2</sup> As soon as the initial temperature was obtained, the substance under investigation was injected either intraperitoneally in the lower left abdominal quadrant or subcutaneously cranio-laterally to the base of the tail. After injection, the animals were kept at room temperature in individual wire cages without food or water. The room temperature varied between 25.5 and 28.5°C.

With the exception of the yeast, which was given as a "15%" autolysate, all test substances were administered in 0.1% solution.

*Results.* Fig. 1 shows the response in temperature following the intraperitoneal injection of 2 cc of a "15%" yeast autolysate contrasted with that after the subcutaneous injection of 2 cc of the same material. The average maximum rise in body-temperature after subcutaneous administration was 1.9°C and appeared at about the 5th hour. After

\* Supported by a grant from the Office of Naval Research.

<sup>1</sup> Baumann, E. J., *J. Biol. Chem.*, 1918, **33**, XIV.

<sup>2</sup> Hill, R. M., Ware, A. G., and Schultz, F. H., *Cancer Research*, 1943, **3**, 839.

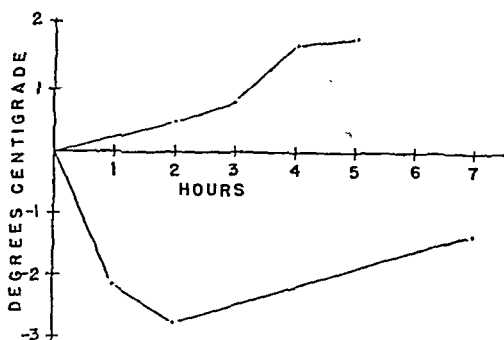


FIG. 1.

Average body-temperature change after administration of 2 cc of "15%" yeast autolysate. Upper curve, 150 rats, subcutaneous injection. Lower curve, 15 rats, intraperitoneal injection.

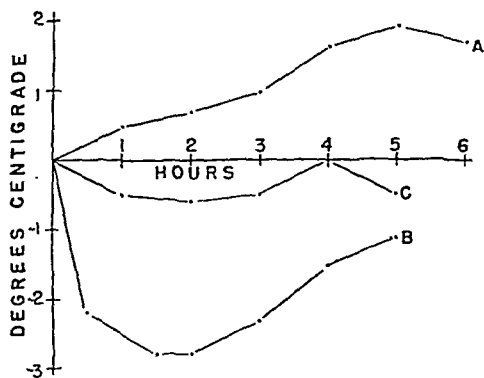


FIG. 2.

Average body-temperature change after administration of 2.5 cc of 0.1% magnesium nucleinate. A, 8 rats, subcutaneous injection. B, 8 rats, intraperitoneal injection. C, average body-temperature change after intraperitoneal injection of 2.0 cc of 0.1% guanine, 8 rats.

intraperitoneal administration, a rapid fall in body-temperature occurred, which reached an average minimum of  $2.7^{\circ}\text{C}$  below the initial value at about the 2nd hour after injection.

In Fig. 2 are shown the results of similar experiments in which 2.5 cc of a 0.1% solution of the magnesium salt of yeast nucleic acid was used. The average maximum rise in body-temperature after subcutaneous administration was  $1.8^{\circ}\text{C}$ , which occurred at about the 5th hour. The average minimum body-temperature after intraperitoneal injection was  $2.9^{\circ}\text{C}$  below the initial value and appeared after about 1.5 hours. The similarity between the results with yeast autolysate and with yeast nucleic acid is striking. Although

2.0 cc of a "15%" yeast autolysate was used on the one hand and 2.5 cc of a 0.1% nucleic acid solution was used on the other, the amount of nucleic acid injected into each animal was about the same, according to the analyses of yeast reported by Von Euler, Ahlstrom and Högberg.<sup>3</sup>

The effect of guanine injected intraperitoneally was slight (see the average curve in Fig. 2) and was not evident in all the animals. Guanine showed no effect when administered subcutaneously. Adenine, uracil, xanthine, and allantoin had no effect on the body-temperature by either route of administration.

During hypothermia, the rats were quiet, perhaps somewhat depressed, and showed a tendency to lie on the side. However, when they were disturbed, they reacted as quickly as untreated animals, and seemed normal in every way.

The rise in body-temperature after subcutaneous injection of autolyzed yeast or the magnesium salt of nucleic acid was slow and the peak was prolonged. The fall after intraperitoneal injection of these substances was rapid, the degree of body-temperature change was greater, and in most instances the return to the initial value occurred in a shorter time. These facts suggest that the difference in response to the two routes of administration may be due to different rates of absorption. However, very small doses given intraperitoneally do not produce hyperthermia, and large subcutaneous injections do not produce hypothermia. Another possibility is that the hypothermia following intraperitoneal injections is a manifestation of shock. Against this is the normal appearance of the peritoneal and other serosal surfaces, and the absence of visceral hyperemia, petechiae, and edema in the animals sacrificed at the time of maximum hypothermia.

*Discussion.* The fact that very small amounts of yeast autolysate or yeast nucleic acid given intraperitoneally did not produce hyperthermia and very large amounts given subcutaneously did not produce hypothermia would seem to rule out differences in the rate

<sup>3</sup> Von Euler, H., Ahlstrom, L., and Högberg, B., *Z. f. physiol. chem.*, 1942, **277**, 1.

and amount of absorption as the cause of the contradictory effects on body-temperature. Our experiments imply that the property of producing hyperthermia after subcutaneous injection and hypothermia after intraperitoneal injection is inherent in the nucleic acid molecule. The fact that the large nucleic acid molecule was active though the smaller hydrolysis products of nucleic acid were not suggests that molecular or particle size may be important in causing body-temperature changes. This raises the questions of phagocytosis of the material in the peritoneal cavity and its absorption by way of the lymph channels, and the possibility that hypothermia is associated with activity of the reticulo-endothelial system. Investigations of these questions are in progress and will be reported later.

**Summary.** Subcutaneous injection of 2 cc of a "15%" yeast autolysate in rats produced a significant rise in body-temperature. Intraperitoneal injection of the same material produced a significant fall in body-temperature. Both qualitatively and quantitatively similar results were produced by the injection of 2.5 cc of a 0.1% solution of the magnesium salt of yeast nucleic acid. Slight lowering of the body-temperature occurred in some animals after intraperitoneal injection of guanine. Subcutaneous injection of guanine had no effect on body-temperature. Adenine, uracil, xanthine, and allantoin had no effect on the body-temperature of rats by either route of administration.

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### 17061. Development of Macrocytic Erythrocytes in Leukemic Subjects Receiving Folic Acid Antagonist, 4-Aminopteroylglutamic Acid (Aminopterin).

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Temporary remissions in acute leukemia have been produced by the therapeutic administration of the folic acid antagonist 4-aminopteroylglutamic acid (aminopterin). Farber<sup>1,2</sup> has reported remissions in children. Dameshek<sup>3,4</sup> has produced equally good results in adults. The exact mechanism of the action of aminopterin is not known. It is a folic acid antagonist in that it possesses the property of inhibiting the growth of *Streptococcus faecalis* R. or *L. casei* in the presence of marginal levels of folic acid. Farber<sup>2</sup> noticed hypersegmentation of neutrophilic granulocytes in the peripheral blood and megaloblasts in the marrow in leukemic patients receiving folic acid antagonists. If aminopterin is a true folic acid antagonist one can theorize that mor-

phologic changes might be produced in the erythrocytes. In a series of 25 patients (children and adults) with acute leukemia of all types treated with aminopterin, results were obtained in 7 of these individuals which support this theory.

**Experimental.** Daily doses of  $\frac{1}{2}$  to 1.0 mg of aminopterin were given intramuscularly. One cc of crude liver containing 2 U.S.P. units was given with each dose of aminopterin.

Frequent complete blood counts were done on all patients receiving therapy. Occasional bone marrow biopsies were also obtained. More complete studies were obtained when macrocytosis and anisocytosis was observed in the circulating red blood cells. These studies included red cell counts, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, morphologic observations on the erythrocytes and measurement of the mean erythrocytic diameter by the

<sup>1</sup> Farber, S., Diamond, L. K., Mercer, R. D., Sylvester, R. F., and Wolff, J. A., *New England J. Med.*, 1948, **238**, 787.

<sup>2</sup> Farber, S., *Blood, J. Hemat.*, 1949, **4**, 160.

<sup>3</sup> Dameshek, W., *Blood, J. Hemat.*, 1948, **3**, 1037.

<sup>4</sup> Dameshek, W., *Blood, J. Hemat.*, 1949, **4**, 168.

TABLE I.

Observations on Erythrocytes in Four Leukemic Patients Having the Most Marked Macrocytosis and Anisocytosis.

Age, yrs.	Sex	Days therapy	Total rbc.	Hgbn., g	MCH, $\gamma\gamma$	Diameter, $\mu$	Macrocytes	Anisocytes
3	M	0	2.57	8.0	30	7.0	0	0
		30	2.91	9.0	31	7.2	++	++
		65	4.10	11.8	29	7.8	++	++
3	F	0	*5.04	14.0	28	7.2	0	0
		90	4.79	13.0	27	7.5	++	++
13	M	0	3.91	10.4	27	7.0	0	0
		15	3.22	9.3	29	7.2	+	+
		30	3.21	9.8	30	7.5	++++	++++
4	F	0	2.20	4.9	22	7.0	0	0
		32	3.78	10.5	28	8.5	++++	++++

\* Transfusions.

Haden-Hausser erythrocytometer.

**Observations.** An obvious macrocytosis and anisocytosis developed in 7 patients. A diagnosis of acute leukemia had been made in each instance by history, examination of the patient, peripheral blood studies and bone marrow biopsies. Although it is realized that cell types in leukemic diseases of children are extremely difficult to classify it is believed that 5 cases were acute lymphatic leukemia and 2 were of the acute monocytic type. Five of the patients were under 10 years of age and 2 were in their teens.

In 4 of the 7 individuals in whom morphologic changes were observed in the red cell series a very marked macrocytosis developed. These data are presented in Table I. In no instance did a severe anemia exist. All had responded well to aminopterin and the leukemic disease had at least been controlled to the extent that no transfusions were necessary. The macrocytosis and anisocytosis in the 7 patients usually became noticeable between the fifteenth and thirtieth days of therapy. Poikilocytosis was not noticed in any of the 7 patients. The mean diameter of the red cells as measured by the erythrocytometer became greater as will be noticed in Table I. Actually an accurate evaluation of the morphologic changes in the red cells could be made by direct microscopic examination of the red cells. Photomicrographs of the red cells in 2 subjects are shown in Fig. 1. The degree of macrocytosis and anisocytosis with-

out poikilocytosis is indeed marked.

Bone marrow biopsies were done in 2 instances when the peripheral blood showed morphologic red cell changes. Even though the peripheral blood had returned to normal and the patients were markedly improved the marrow tissue was still markedly infiltrated and replaced with leukemic cells to the extent that only rare normal bone marrow cellular elements were observed. No true megaloblasts were observed in these 2 patients. In other individuals however who have had extensive therapy with aminopterin an exceedingly rare megaloblast has been observed.

**Discussion.** Farber<sup>1</sup> in his initial report emphasized the toxic nature of aminopterin. Many of the lesions produced are similar to those produced by a folic acid deficiency in the rat and monkey. These lesions are stomatitis, ulceration of the mucous membrane of the mouth, smooth tongue, pharyngitis, and atrophic changes in the intestinal epithelium. The morphologic changes in the erythrocytes consisting of macrocytosis and anisocytosis may be possibly due to an induced folic acid deficiency. It is of interest that poikilocytosis is not present in the 7 cases reported herein. Farber<sup>2</sup> and Dameshek<sup>3,4</sup> both emphasize that it is impossible to state at this time whether all the changes produced in acute leukemia by folic acid antagonists are primarily due to a folic acid deficiency. When untoward symptoms rapidly develop that are similar to those produced experimentally, the various types of

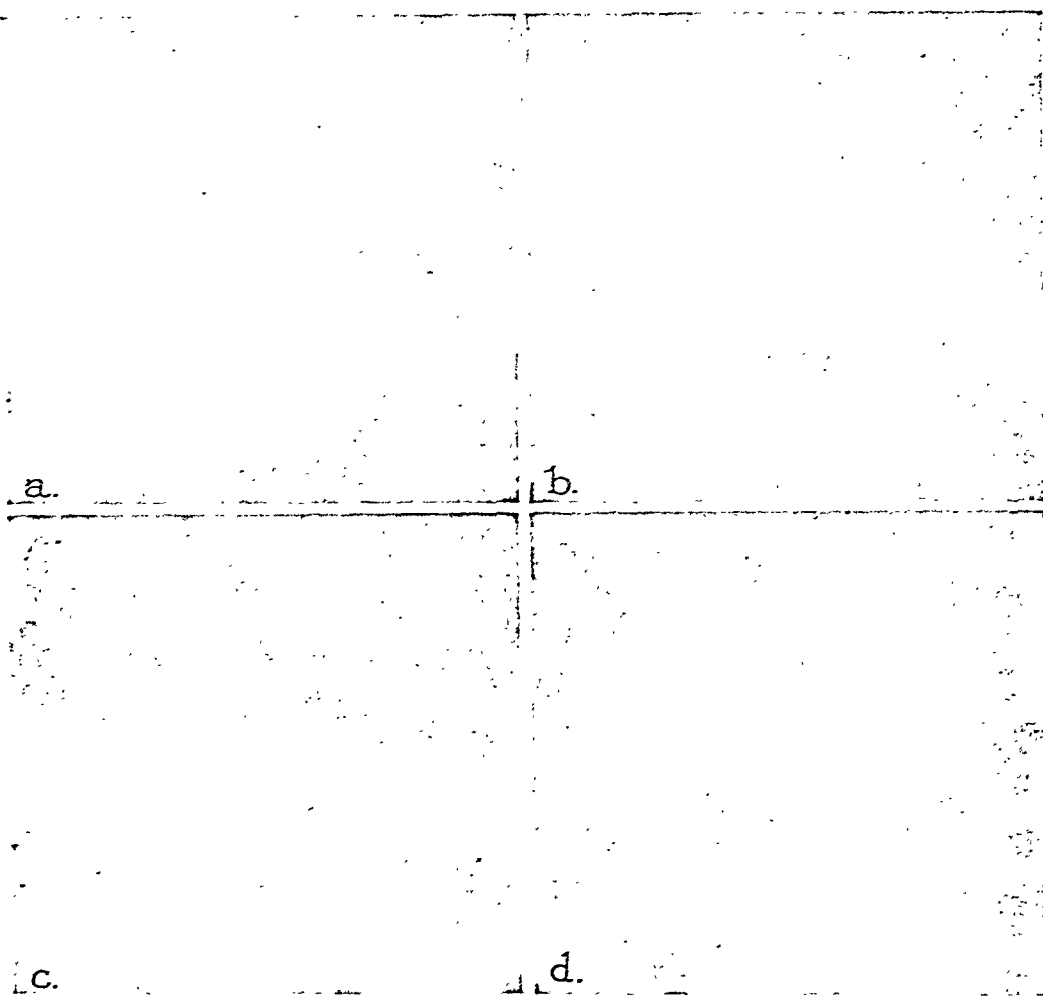


FIG. 1.

Morphologic changes in red blood cells during aminopterin therapy. A and b, patient 48-279. a, before therapy, and b, after therapy. C and d, patient 48-229. c is before therapy and d, after therapy. In each instance macrocytosis and anisocytosis are present after therapy.

therapy such as vitamin B preparations, liver extracts both crude and concentrated, and folic acid have not been more beneficial than the mere act of stopping the administration of the folic acid antagonist. Further observations on complicated biochemical systems must be made before definite conclusions can be reached.

**Conclusions.** In 7 of 25 human subjects with acute leukemia treated with the folic acid antagonist 4-aminopteroylglutamic acid (aminopterin) morphologic changes developed in the circulating red blood cells consisting of a macrocytosis and anisocytosis.

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## 17062. Effect of Heparin on Artificial Activation in the Frog Egg.\*

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It is a well known fact that protoplasmic gelation precedes mitosis. The colloidal theory of cell division likens this protoplasmic gelation to blood clotting (see Heilbrunn<sup>1</sup> for discussion). It would be expected, therefore, on the basis of this theory that anticoagulants, such as heparin, might prevent protoplasmic gelation. Indeed, Heilbrunn and Wilson<sup>2</sup> have shown that this gelation in the Chaetopterus egg can be prevented by heparin and, moreover, that cell division is subsequently inhibited. Heparin also inhibits cell division in tissue cultures (Fischer<sup>3</sup>).

The most effective method of producing artificial parthenogenesis in the frog egg is by pricking the egg in the presence of blood or blood serum (Bataillon<sup>4</sup>). It was thought that the active principle in the blood might

be a thrombin or thrombin-like substance which could initiate a protoplasmic gelation resulting in cell division. If this were true, anticoagulants should have some effect on parthogenesis in preventing the action of blood serum. The following experiments indicate that such is indeed the case.

*Rana pipiens* females were induced to ovulate by the implantation of pituitary glands, and the ovulated eggs were stripped in rows on to glass slides immediately before use. The eggs were covered with the blood mixture to be tested, and then pricked with a fine glass needle. For a discussion of the technic employed see Rugh.<sup>5</sup> Mixtures consisting of one part frog blood collected by heart puncture to one part heparin solution were employed in the above procedure. The heparin<sup>1</sup>

TABLE I.  
% Cleavage of Eggs Pricked in 1:1 Mixtures of Blood and Heparin Made Up in Ringer's Solution.

		Concentration of Heparin in g %					
	0.0	0.2	0.4	0.8	1.6	2.4	3.2
68		60	—	—	—	—	—
41		—	25	19	—	—	—
63		—	—	54	42	—	—
46		38	40	43	24	—	—
62		56	55	56	46	—	—
70		—	—	51	50	—	56
67		—	—	—	66	—	63
72		—	—	69	72	—	65
61		—	—	44	48	—	44
64		—	—	40	43	46	34
49		42	48	—	—	—	—
61		—	45	38	45	48	32
76		63	74	—	—	69	62
77		72	67	—	—	65	55
77		—	—	—	—	66	—
67		61	65	—	—	63	60
59		39	23	—	—	31	—
68		68	54	—	—	60	—
51		29	—	—	—	26	—
43		—	—	—	—	32	—
64		—	—	36	45	—	38

\* Aided by a grant from the U. S. Public Health Service, administered by L. V. Heilbrunn.

<sup>1</sup> Heilbrunn, L. V., *An Outline of General Physiology*, 2nd ed., Philadelphia, 1943.

<sup>2</sup> Heilbrunn, L. V., and Wilson, W. L., *Proc. Soc. Exp. Biol. and Med.*, 1949, **70**, 179.

<sup>3</sup> Fischer, A., *Arch. f. path. Anat. u. Physiol.*, 1930, **279**, 94.

<sup>4</sup> Bataillon, E., *Compt. rend. Acad. Sci. Paris*, 1911, **152**, 920.

<sup>5</sup> Rugh, R., *Experimental Embryology*, New York, 1941.

was made up in frog Ringer's solution in concentrations of 0.2, 0.4, 0.8, 1.6, 2.4, and 3.2 grams percent. Control eggs were covered with a mixture of one part frog blood to one part Ringer's. A third set of eggs were pricked in the absence of any solution. In this way it was possible to compare the effectiveness in producing artificial parthenogenesis of heparinated blood with the effectiveness of plain blood of the same concentration. After treatment the eggs were immediately transferred to pond water which had been boiled and filtered, and were kept at room temperature. Four to 5 hours later, counts were made of the number of divided cells and the total number of cells. No attempt was made to distinguish between normal and abnormal divisions.

The results are summarized in Table I, which shows the percent cleavage obtained when the eggs were pricked in the presence of blood and Ringer's fluid (0% heparin in column 1), and in the presence of blood and heparin of various concentrations (columns 2-7). In every case the percent of dividing eggs was lower when the heparinated blood was used than when the blood-Ringer's solution was used. In only one case, however, was the percent as low as in eggs treated by pricking alone. The values obtained for the experimental eggs were calculated as percent

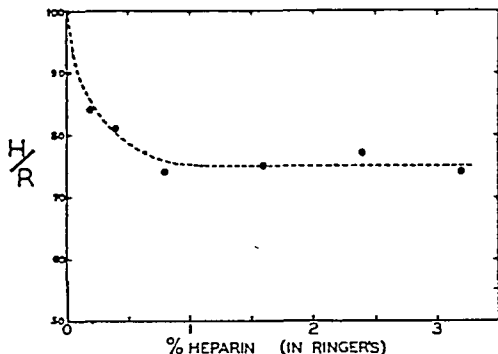


FIG. 1.

Inhibition by Heparin of Artificial Activation.

$$\frac{H/R}{\% \text{ Cleavage in Blood-Ringer's Solution}} \times 100$$

of the blood-Ringer's controls, averaged for each concentration, and they were then plotted in Fig. 1. The 100% line represents the cleavages in the blood-Ringer's fluid. From this figure it can be seen that there is a definite inhibition by heparin of activation by pricking in the presence of blood. This lends further support to the colloidal theory of cell division.

**Summary.** Eggs of *Rana pipiens* were pricked in the presence of heparinated frog blood. The percent of dividing eggs was lower in every case than when the eggs were pricked in the presence of blood and Ringer's fluid

† Hynson, Westcott and Dunning, lots 198 and 202.

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## 17063. Failure of Rutin to Inhibit Hyaluronidase in the Albino Rat.

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Rutin, a naturally occurring flavanol glucoside, has been found to influence favorably various states characterized by an increased capillary fragility.<sup>1</sup> It has been demonstrated that hyaluronidase increased the speed of passage of fluids and the blue dye T-1824 across

the capillary membrane in the albino rat.<sup>2</sup> Chambers and Zweifach<sup>3</sup> have considered that hyaluronidase accentuated capillary fragility rather than produced direct changes in capillary permeability. *In vitro*, rutin in high

<sup>1</sup> Griffith, J. Q., Jr., Couch, J. F., and Lindauer, M. A., *Proc. Soc. Exp. Biol. and Med.*, 1944, 55, 223.

<sup>2</sup> Elster, S. K., Freeman, M. E., Dorfman, A., *Am. J. Physiol.*, 1949, in press.

<sup>3</sup> Chambers, R., and Zweifach, B. W., *Physiol. Rev.*, 1947, 27, 436.

concentrations effectively inhibited hyaluronidase, but was inactive in lower concentrations.<sup>4</sup> Levitan<sup>5</sup> tested this reaction *in vivo* in the albino rat, and concluded that rutin markedly inhibited the spreading activity of intradermally injected inked hyaluronidase.

A technic has been described<sup>6</sup> in which the activity of hyaluronidase was tested in the albino rat. Following the intravenous administration of hyaluronidase, changes in blood volume occurred. Edema of the extremities appeared and marked elevation of the blood hematocrit was noted. A substance may be tested for its ability to inhibit this reaction, if it does not of itself alter the fluid balance of the body. Thus, a substance may be considered a hyaluronidase antagonist if, after its administration, the hematocrit does not rise following the injection of the standard amount of enzyme.

This technic has been applied to the study of rutin in the body and its possible role in hyaluronidase inhibition.

**Experimental.** 136 male albino rats of the Sherman strain were fed Purina laboratory chow and water *ad lib*. The hyaluronidase was prepared according to the method of Freeman *et al.*<sup>7</sup> The enzyme was assayed<sup>8</sup> and measured 3000 turbidity reducing units (T.R.U.) per mg of nitrogen.

1) *The effect of the route and mode of administration of rutin on the hematocrit.* 64 animals, weighing 200-350 g were divided into 5 groups:

Group A—20 normal rats.

Group B—15 rats, 200 mg rutin suspended in 1 ml normal saline fed orally (via stomach tube) for 4 successive days.

Group C—9 rats, 200 mg rutin suspended in 1 ml normal saline injected intraperitoneally.

Group D—10 rats, 1 ml propylene glycol injected intraperitoneally.

Group E—10 rats, 200 mg rutin dissolved in 1 ml propylene glycol injected intraperitoneally.

All animals were bled from the aorta and sacrificed. The hematocrit was determined by centrifugation at 3000 rpm for 30 minutes.<sup>9</sup> Blood was drawn from Groups C, D and E 3½ hours following the injections and from Group B 1½ hours following the last gastric administration.

Marked changes were noted in the animals in Groups C, D and E. They were lethargic and their abdomens were protuberant. When their abdominal cavities were opened, several ml of free fluid were encountered. In Groups C and E, this fluid was light yellow; rutin was precipitated on the peritoneal surfaces of the organs. The abdominal fluid encountered in the animals of Group D was slightly cloudy and colorless. The intestines and peritoneum were inflamed and edematous. The most severe changes were noted in the animals of Group E, in which as much as 8 ml of fluid were removed from the abdominal cavity. The animals of Groups A and B appeared normal.

The changes in plasma volume are reflected in the hematocrit determinations (Fig. 1).

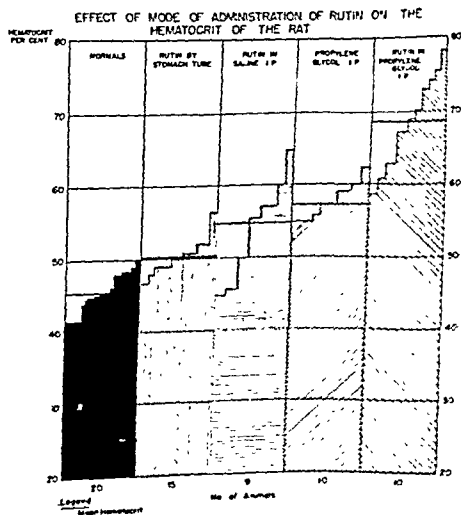


Fig. 1.

<sup>4</sup> Beiler, J. M., and Martin, G. J., *J.B.C.*, 1947, 171, 507.

<sup>5</sup> Levitan, B. A., *Proc. Soc. Exp. Biol. and Med.*, 1948, 68, 566.

<sup>6</sup> Elster, S. K., Freeman, M. E., Anderson, P., *J. Lab. and Clin. Med.*, 1949, in press.

<sup>7</sup> Freeman, M. E., Anderson, P., Oberg, M., and Dorfman, A., 1949, in press.

<sup>8</sup> Dorfman, A., and Ott, M. L., *J.B.C.*, 1948, 172, 367.

<sup>9</sup> Wintrobe, M. M., *Clinical Hematology*, Lea & Febiger, 1947, Philadelphia.

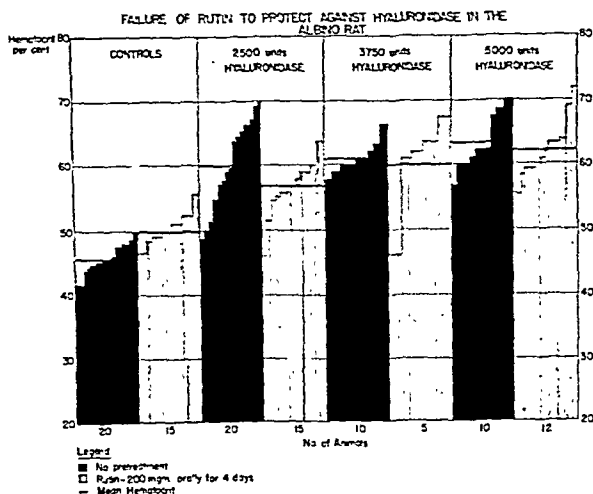


FIG. 2.

The mean hematocrit of Group A, 46.0%, was representative of normal values obtained for a similarly constituted normal population of 150 male rats. All other groups showed evidence of hemoconcentration. The mean hematocrit value for Group C was 54.4%, Group D 57.1% and Group E 68.5%. A few animals of Group C had a normal hematocrit, but none of Group D or E was normal. Group E had the greatest clinical effects and the greatest hemoconcentration. Every animal of this group had severe diminution of plasma volume. The hematocrits of the rats in Group B were slightly elevated; the mean value was 50.3%. Most animals of this group had little or no change of hematocrit and only one had a moderate increase (56%). Therefore, the oral route was selected as the best available means of rutin administration for the second part of the experiment, since it caused the least change in the hematocrit.

2) *The role of rutin in hyaluronidase antagonism in vivo.* 72 animals weighing 160-230 g were used. Thirty-two animals were fed 200 mg rutin in 1 ml saline by stomach tube for 4 successive days. On the last day 1½ hours following the gastric administration, 15 rats were given 2500 T.R.U., 5 rats were given 3750 T.R.U. and 12 animals were given 5000 T.R.U. hyaluronidase by the intravenous route. At the end of 30 minutes, they were bled from the aorta and the hematocrit

of the blood determined. Forty animals were not pretreated with rutin; 20 rats were given 2500 T.R.U., 10 rats administered 3750 T.R.U. and 10 were given 5000 T.R.U. hyaluronidase intravenously. These were bled and sacrificed 30 minutes after the injection and the blood hematocrit determined.

The animals that received hyaluronidase alone, developed edema of the extremities and face within 5-15 minutes; this was maximal in 30 minutes. In a previous communication<sup>2</sup> it has been reported that the edema began to recede in 2 hours and had disappeared completely in 24 hours. The hematocrits of the blood were markedly elevated (Fig. 2); the mean values were 60.0 to 62.9%. The group that was pretreated with rutin and then received hyaluronidase intravenously was indistinguishable from the previous group. Edema of equal intensity and duration appeared in the same areas. The hematocrits of these animals were elevated and the mean values were not significantly different from that group receiving hyaluronidase alone. Although the range of values was different, the mean hematocrit (57.1%) of the animals receiving 2500 units hyaluronidase and rutin was not statistically different from the mean (60.0%) of the group receiving only hyaluronidase. In the groups receiving the larger amounts of hyaluronidase, only one animal failed to respond with a rise of hematocrit.

**Discussion.** In the evaluation of the inhibitory action of rutin on hyaluronidase *in vivo*, the method of rutin administration must be considered. It has been demonstrated that 200 mg rutin suspended in saline, when given intraperitoneally to a rat, acted as an irritant and caused the formation of hydroperitoneum. Propylene glycol had a similar action. Rutin dissolved in propylene glycol was the most active and caused so much peritoneal fluid loss that marked hemoconcentration occurred. In the experiments of Levitan, 200 mg rutin dissolved in 1 ml propylene glycol were given intraperitoneally to rats weighing 250-400 g. Three and one-half hours later, testicular hyaluronidase and india ink were injected into the skin and the rats were sacrificed 22 hours after that. There was marked inhibition of the spread of the hyaluronidase-ink mixture, as well as a decrease in the spread of the control ink and saline. Propylene glycol alone, when given intraperitoneally, caused a smaller decrease in the spread of the hyaluronidase. Since both of these agents have been demonstrated to cause extreme hemoconcentration when given intraperitoneally, the validity of conclusions as to the efficacy of these drugs on hyaluronidase inhibition is open to extreme doubt. Duran-Reynals<sup>10</sup> has emphasized that "in the living animal a permeating effect on blood capillaries takes place which may contribute to the final phase of the spreading." In an animal in a condition of marked diminution of blood volume, the available fluid for

the spreading reaction is greatly diminished and the conditions for spread are not optimal.

Rutin has been found to inhibit hyaluronidase *in vitro*, and this inhibition has been potentiated by Vitamin C. These studies have demonstrated that rutin, when administered orally, failed to modify the action of hyaluronidase on edema formation and elevation of hematocrit in the albino rat. Rutin in the body is presumed to decrease capillary fragility. However, the investigations performed at this laboratory have failed to produce evidence that hyaluronidase increased capillary fragility, as the edema is not accompanied by red blood cell extravasation.

**Summary.** 1. Hyaluronidase increased the diffusion of fluid across the capillary membrane and cause elevation of the blood hematocrit.

2. Rutin, in the amounts used in these experiments, failed to inhibit this reaction in the albino rat.

3. Rutin in propylene glycol, given intraperitoneally to the albino rat, caused the formation of hydroperitoneum and marked hemoconcentration. Propylene glycol given by the same route had a similar, although lesser, effect. When administered orally, rutin caused little elevation of the hematocrit.

The assistance of Miss Elaine Lowry is gratefully acknowledged. The author is indebted to Major Monroe E. Freeman, MSC, for the hyaluronidase preparations.

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<sup>10</sup> Duran-Reynals, F., *Bact. Rev.*, 1942, 6, 197.

## 17064. Role of Potassium in Dialysing Fluid in Treatment with the Artificial Kidney.

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Spontaneous elevations of serum potassium levels with electrocardiographic changes simi-

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† Archibald Fellow in the Department of Experimental Surgery, McGill University.

lar to those found in dogs with experimental anuria<sup>1</sup> have been demonstrated in man during the terminal stages of uremia,<sup>2-6</sup> indicating that hyperpotassemia may be an important

<sup>1</sup> Hoff, H. E., Smith, P. K., and Winkler, A. W., *J. Clin. Invest.*, 1941, 20, 607.

TABLE I.  
Concentration of Potassium in Patient's Serum and in the Dialysing Fluid Before and After Dialysis.

Case No.	Serum K		Length of treatment, hrs	K of dialysing fluid		Amt of K removed by dialysis, g
	Before dialysis, mg %	After dialysis, mg %		Before treatment, mg %	After treatment, mg %	
I	26.3	23.7	6	20.1	20.9	.80
II	28.3	24.6	6	5.0	12.6	7.6
III	20.4	11.9	6*	7.76	7.56†	—

\* After 4 hours 35 g of KCl was added to the dialysing fluid because of the changes in the electrocardiogram, indicating hypopotassemia. The potassium content of the dialysing fluid was hereby increased to 24.5 mg %. Treatment was then continued for another 2 hours.

† Potassium content of the dialysing fluid after the first 4 hours of treatment before the 35 g of KCl was added.

factor in the mechanism of death in uremia, especially when associated with anuria or oliguria.

This study was undertaken to estimate the use of an artificial kidney in the elimination of potassium from the body in cases of uremia in which the serum potassium levels are elevated.

**Methods.** Serum potassium was determined and electrocardiograms were taken in 3 patients with a severe degree of uremia, prior to, during, and after treatment with the artificial kidney. The artificial kidney and the constitution of the dialysing fluid were similar to those used by Kolff,<sup>7</sup> but in cases II and III no KCl was added to the dialysing fluid. The potassium in the dialysing fluid was estimated before and after treatment. Its level of 5 and 7.76 mg% in the dialysing fluid before treatment in cases II and III respectively (Table I) was probably due to potassium contaminants in the other salts and the tap water in the dialysing fluid. The other biochemical and clinical findings in these cases will be reported in detail elsewhere.<sup>8,9</sup>

**Results.** From the experimental data in

<sup>2</sup> Marchand, J. F., and Finch, C. A., *Arch. Int. Med.*, 1944, **73**, 384.

<sup>3</sup> Keith, N. M., Burchell, H. B., and Baggenstoss, A. H., *Am. Heart J.*, 1944, **27**, 817.

<sup>4</sup> Stewart, H. J., Shepard, E. M., and Horger, E. L., *Am. J. Med.*, 1948, **5**, 821.

<sup>5</sup> Bywaters, E. G. L., *J.A.M.A.*, 1944, **124**, 1103.

<sup>6</sup> Strauss, M. B., *New England J. Med.*, 1948, **239**, 693.

<sup>7</sup> Kolff, W. J., "New Ways of Treating Uremia," J. & A. Churchill, Ltd., London, 1947.

Table I, it is apparent that when the potassium content of the dialysing fluid is below 20 mg% (see case II) a significant amount of potassium can be removed by artificial dialysis from the body of uremic patients with hypopotassemia, without danger of hypopotassemia. When the potassium of the dialysing fluid is maintained at 20.1 mg% (case I) the serum potassium is lowered only slightly and an insignificant amount of potassium is actually removed from the body. In uremic patients with normal serum potassium levels, hypopotassemia can occur when the potassium of the dialysing fluid is markedly lowered (case III, Table I).

In case III, changes in the electrocardiogram indicative of some degree of hypopotassemia<sup>10</sup> were observed after 4 hours of dialysis and potassium was then added to the dialysing fluid and the dialysis continued for another 2 hours. Although the serum of the blood withdrawn at this time was found to contain 11.9 mg% of potassium, the patient did not show clinical symptoms associated with hypopotassemia. During the dialysis the patient voided 1,200 cc of urine. The serum potassium level 5 hours after the dialysis was 14.6 mg%.

**Discussion.** Kolff<sup>7</sup> originally omitted potassium from the dialysing fluid, but owing

<sup>8</sup> Miller, G. G., and Ripstein, C. B., to be published.

<sup>9</sup> Wener, J., de Leeuw, N. K. M., and MacLean, J. T., to be published.

<sup>10</sup> Martin, H. E., and Wertman, M., *Am. Heart J.*, 1947, **34**, 646.

to the possible dangers of producing hypopotassemia, he and later Bywaters and Joekes,<sup>11</sup> both recommended that physiological amounts of potassium be included in the dialysing fluid.

From Kolff's<sup>7</sup> data it was apparent that hypopotassemia occurred only in a patient who had a normal serum potassium concentration to start with, very similar to that in case III, Table I.

The variable results obtained in these cases are probably due to the differences in the direction of the potassium shift. In case II, the increase of 7.6 g of potassium in the dialysing fluid during the dialysis indicated that potassium was removed from the intracellular as well as from the extracellular fluids. In cases I and III, since there was no significant increase of potassium in the dialysing fluid, the potassium shift probably occurred mainly from the extracellular to the intracellular fluids. The lowering of the serum potassium in these instances might be explained by other factors: (a) the dilution of the patient's blood with the blood added to the artificial kidney prior to its use, and by the further addition of varying amounts of blood and intravenous fluids during the treatment: (b) the fact that the glucose present in very high amount<sup>7,11</sup> in

the blood leaving the artificial kidney to be returned to the body will, on entering the systemic circulation, be stored as glycogen, with the resultant shift of potassium from the extracellular to the intracellular spaces<sup>10</sup> was the main reason that Bywaters and Joekes<sup>11</sup> recommended that physiological amounts of potassium to be added to the dialysing fluid; and (c) potassium elimination from the blood stream by urinary excretion. This last factor may have operated in Case III where the patient voided 1,200 cc urine during the period of dialysis.

**Summary.** From the observations reported it would appear that the artificial kidney can serve as a safe and efficient method for removing excess potassium from the body in uremia patients with hyperpotassemia. However, further study is required to determine the amount of potassium that should be added to the dialysing fluid in each individual case. This will probably vary with the degree of hyperpotassemia present before treatment is begun.

We wish to express our thanks to Doctors G. G. Miller and J. T. MacLean for permission to study their patients. We are grateful to Dr. Martin Hoffman for many helpful suggestions and criticisms.

<sup>11</sup> Bywaters, E. G. L., and Joekes, A. M., *Proc. Royal Soc. Med.*, 1948, **41**, 420.

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## 17065. Nu 445 in the Treatment of Urinary Infections Due to Gram Negative Bacilli.\*

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**Introduction.** Nu 445<sup>†</sup> (3,4-dimethyl-5-sulfanilamido-isoxazole) is one of the newer sulfonamides said to be highly effective for urinary tract infections due to *E. coli* and *B. proteus*.<sup>1-6</sup> The reported results on the thera-

peutic activity of this drug in man are meager

<sup>1</sup> Schuitzer, R. J., Foster, H. K., Ercoli, N., Soo-Hoo, A., Mangieri, C. N., Roe, M. D., *J. Pharm. and Exp. Therap.*, 1946, **88**, 47.

<sup>2</sup> Sarnoff, S. J., *Proc. Soc. Exp. Biol. and Med.*, 1948, **68**, 23.

<sup>3</sup> Narins, L., *J. Urol.*, 1948, **59**, 92.

<sup>4</sup> Sarnoff, S. J., Freedman, M. A., Hyman, A. A., *J. Urol.*, 1946, **55**, 417.

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<sup>†</sup> Supplied through the courtesy of Hoffmann-La Roche, Inc.

TABLE I.

Comparative *in vitro* Sensitivity of Several Strains of Various Gram Negative Micro-organisms to 4 Sulfonamides.

Strain	Nu 445† titers in mg %		ST‡ titers in mg %		SD‡ titers in mg %		SMT‡ titers in mg %	
	c*	st†	c	st	c	st	c	st
<i>E. coli</i> 1	750	500	500	100	250	50	25	25
" 2	1000	500	500	100	250	100	250	100
" 3	750	500	1000	1000	1000	250	50	25
" 4	100	25	500	250	500	250	750	100
" 5	250	100	500	100	500	25	500	250
<i>A. aerogenes</i> 1	750	500	500	100	>1000	>1000	750	500
" 2	>1000	>1000	1000	750	>1000	1000	250	100
" 3	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000
" 4	1000	500	500	100	>1000	1000	750	500
<i>B. proteus</i> 1	>1000	500	750	50	250	25	100	25
" 2	>1000	>1000	1000	500	>1000	>1000	500	100
" 3	>1000	>1000	>1000	>1000	>1000	>1000	1000	750
" 4	250	100	1000	500	>1000	>1000	750	250
<i>B. pyocyaneus</i> 1	>1000	>1000	>1000	1000	>1000	>1000	750	500
" 2	500	100	>1000	750	>1000	>1000	1000	500
" 3	>1000	>1000	500	100	>1000	>1000	750	500
" 4	>1000	1000	>1000	>1000	>1000	>1000	500	100
<i>E. typhi</i> 1	1000	250	500	250	1000	500	250	50
" 2	750	250	500	100	1000	500	500	100
<i>S. paratyphi</i> 1	1000	250	500	250	>1000	>1000	250	25
" 2	1000	250	750	500	>1000	>1000	250	100
<i>S. schottmuelleri</i> 1	>1000	1000	>1000	750	>1000	>1000	500	100
" 2	>1000	1000	>1000	750	>1000	750	500	100

\* c = bactericidal titer.

† st = bacteriostatic titer.

‡ NU 445—3,4-dimethyl-5-sulfanilamido-isoxazole.

ST —sulfathiazole.

SD —sulfadiazine.

SMT —sulfamethazine.

and conflicting. *In vitro* and *in vivo* studies on animals showed that sulfadiazine was slightly superior to Nu 445 against *E. coli*, moderately superior against *S. schottmuelleri* and greatly superior against *Klebsiella A.*<sup>1</sup> Because of these inconsistencies, a further clinical and *in vitro* study of the drug is herewith reported.

*In vitro* studies: The *in vitro* action of the drug on various gram negative bacilli was determined and compared with that of sulfadiazine (SD), sulfamethazine (SMT), and sulfathiazole (ST).

Serial dilutions of a 1% solution of the

<sup>1</sup> Rodgers, R. S., Colby, F. H., *J. Urol.*, 1948, 59, 659.

<sup>2</sup> Haines, W. H., Micelli, S., *Penn. Med. J.*, 1947, 50, 1328.

drug in nutrient broth (pH 8.2) were made, and to 0.9 cc of each dilution was added 0.1 cc (500 to 3000 microorganisms) of a bacterial suspension from a 24-hour growth. The highest drug dilution at which there was no macroscopically visible growth after 24 hours at 37° was taken to be the bactericidal titer (c). The highest drug dilution in which there was definitely less turbidity as compared to the control tubes was taken to be the bacteriostatic titer (st).

Most of the bacterial strains tested were freshly isolated from infected urines. *E. typhi* and the *Salmonellae* had been cultured from stools or blood and had undergone a number of transfers.

Table I shows that the 4 sulfonamides test-



TABLE II.  
Results of Treatment with Nu 445 in Uncomplicated\* and Complicated† Urinary Infections.

Type of disease	Type of infection	No. of cases	Cases cured, No.	Cases not cured, No.
Uncomplicated	Monovalent	7 }	6	3
	Polyvalent	2 }		
Complicated	Monovalent	1 }	1	10
	Polyvalent	10 }		
Total		20	7	13

\* Uncomplicated means acute cystitis, acute pyelitis, or acute pyelonephritis without any additional pathology.

† Complicated means additional pathology such as severe chronic infection, calculi, hydronephrosis, obstruction to urinary flow, or constant drainage.

ed exert differing effects on various strains of the same species of bacteria. For example, of the 5 *E. coli* strains tested, 2 were most sensitive to SMT, 2 to Nu 445, and one equally to SD and SMT. Of 4 strains of *Ps. aeruginosa*, (*B. pyocyaneus*), 3 were best inhibited by SMT, one by Nu 445. The effect of SMT on the typhoid-paratyphoid group was definitely superior to that of the other 3 drugs. From the foregoing data, Nu 445 appeared to be worthy of clinical trial in infections due to *E. coli*, *B. proteus*, or *Ps. aeruginosa*.

**Clinical Observations.** Twenty unselected patients with a variety of urinary tract infections due to gram negative bacilli were given Nu 445 orally. In spite of large daily doses, the blood levels remained rather low indicating rapid acetylation and excretion of the drug.<sup>5</sup>

The results of treatment are indicated by 2 types of response. A. Clinical and bacteriological cure. This result was obtained in 7 patients (35%). The patients in this group were followed for at least 2 weeks, and at least 2 sterile cultures were obtained after termination of treatment. B. Clinical and bacteriological failure. Of the 13 patients (65%) in this group, 3 showed temporary improvement, but had a recurrence shortly after the drug was omitted.

**Discussion.** The results of treatment with Nu 445 are inferior to those obtained with SMT and other sulfonamides.<sup>7-12</sup> Six of 7 patients with pure *E. coli* infection and one

patient with pure *B. proteus* infection were cured. Four of these had received SD without benefit. Not one of 12 patients with mixed infection was cured. Three, however, for various reasons, did not receive a full course of the drug. Ten of the 12 had complicating pathology such as obstruction, calculi, hydronephrosis or constant drainage, etc. (Table II).

Although alkali therapy was not administered, the urine pH ranged from 4.5-7.5. No renal damage or crystalluria was observed in any patient. In 2 patients a dose of 12 g daily for several days and in one patient a dose of 19 g inadvertently given in one day, did not produce any toxic symptoms. Mild nausea and vomiting occurred once after 6 g had been given daily for 4 days, and disappeared within 24 hours after termination of therapy.

Nu 445 is a safe and effective drug in uncomplicated monovalent infections of the urinary tract due to *B. proteus* or *E. coli*, but is of little or no value in polyvalent infections, and in those associated with complicating organic disease in which SD<sup>6</sup> and SMT<sup>7</sup> have occasionally achieved cure. However, Nu 445, like other sulfonamides and antibiotics, may produce a cure when other drugs do not.

**Summary.** 1. An *in vitro* study of the action of Nu 445 shows that this drug occasionally

<sup>9</sup> Satterthwaite, R. W., Hill, J. H., and Young, H. H., *J. Urol.*, 1941, 46, 101.

<sup>10</sup> Helmholtz, H. F., *Proc. Staff Meetings, Mayo Clinic*, 1942, 17, 529.

<sup>11</sup> Culp, O. S., *J. Urol.*, 1940, 44, 116.

<sup>12</sup> Alyea, E. P., *J. Urol.*, 1942, 47, 219.

<sup>7</sup> Rutenburg, A. M., Schweinburg, F. B., *Surgery*, 1949.

<sup>8</sup> LaTowsky, L. W., *J. Urol.*, 1943, 50, 623.

exerts a greater bacteriostatic and bactericidal effect on certain strains of gram negative bacilli than do other sulfonamides.

2. Clinical trial in 20 patients with urinary infections indicated excellent effectiveness of the drug in uncomplicated, monovalent infections due to *E. coli* or *B. proteus*.

3. Results of treatment in polyvalent infec-

tions and in cases with complicating pathology were poor.

4. The marked lack of toxicity of Nu 445 recommends its use in appropriate cases, in which it will occasionally be found to be more effective than other sulfonamides.

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## 17066. Metabolic Fate of 4,4'-Diaminodiphenylsulfone (DDS) in the Rabbit and its Isolation from Urine.

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4,4'-Diaminodiphenylsulfone (DDS) and many of its derivatives have been used with a measure of success in the treatment of experimental tuberculosis.<sup>1,2</sup> More recently the potentiating action of some sulfone derivatives on streptomycin has also been shown.<sup>3</sup> Of the various types of sulfone derivatives indirect evidence has indicated that certain disubstituted derivatives like promin, diasone and possibly sulphetrone are metabolized to the toxic parent substance DDS while the monosubstituted alkyl and hydroxyalkyl derivatives do not appear to undergo such transformation in the body to an appreciable extent.<sup>4</sup> Indirect evidence has also indicated that DDS itself does not change to any significant degree in the body, at least in so far as acetylation is concerned.<sup>1,5</sup> Obviously, direct evidence on these points can only be

had by the actual isolation of DDS from the urine. The present report describes a satisfactory method for the isolation and recovery of DDS added to rabbit urine and the isolation and the identification as DDS of about 70% of the total diazotizable material in the urine of rabbits given DDS orally.

*Procedure.* The isolation of DDS from rabbit urine is based on the solubility of its hydrochloride in water and the relative insolubility of the base in water, but its ready solubility in ethyl acetate. Ethyl acetate appears to be the most satisfactory of several water-immiscible solvents tried.

The urine is made strongly alkaline to litmus with NaOH and chilled on ice for a while to aid precipitation of the base. It is then shaken out thoroughly 2 to 3 times in a separatory funnel with 30 to 50 cc of ethyl acetate, allowing as complete separation as possible each time. The partly emulsified combined ethyl acetate extract is cleared by the gradual addition of anhydrous sodium sulfate with stirring. The clear ethyl acetate solution is decanted and the Na<sub>2</sub>SO<sub>4</sub> residue is washed 2 to 3 times with small portions of ethyl acetate.

The combined ethyl acetate extracts are washed once with about 5 cc H<sub>2</sub>O and then thoroughly extracted with 5 cc portions of N HCl. This should be repeated 5 to 7 times. The combined acid extract is chilled on ice

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<sup>1</sup> Smith, M. I., Emmart, E. W., and Westfall, B. B., *J. Pharm. Exp. Therapy.*, 1942, **74**, 163.

<sup>2</sup> Smith, M. I., *N. Y. State J. Med.*, 1945, **45**, 1665.

<sup>3</sup> Smith, M. I., McClosky, W. T., Jackson, E. L., and Bauer, H., *Proc. Soc. Exp. Biol. and Med.*, 1947, **64**, 261.

<sup>4</sup> Smith, M. I., Jackson, E. L., Junge, J. M., and Bhattacharya, B. K., *Am. Rev. Tuberc.*, in press.

<sup>5</sup> Smith, M. I., and McClosky, W. T., *Am. Rev. Tuberc.*, 1945, **52**, 304.

TABLE I.

Fractionation of Rabbit Urine for the Isolation of 4,4'-Diaminodiphenylsulfone (DDS). Figures in first 4 fractions give DDS equivalent in mg as obtained by the Bratton and Marshall Diazotization Technic.

Fraction	Urine of rabbits given 0.5 g/kg DDS orally			
	30 cc normal urine with 63 mg DDS added	30 cc urine containing 100 mg DDS equivalent	30 cc urine containing 100 mg DDS equivalent	60 cc urine containing 200 mg DDS equivalent
1. Residual urine after extraction with ethyl acetate	0.25	7.2	8.8	10.3
2. Ethyl acetate ext. after extraction with N HCl evaporation to dryness, residue taken up in dil HCl and filtered	0.25	4.8	5.8	7.1
3. Mother liquor and washings of crystals obtained by alkalization of the N HCl extract	9.0	20.3	15.6	20.9
4. Acetone insoluble part of isolated crystals	trace	trace	trace	—
5. Acetone soluble part of isolated crystals				
(a) Weight mg	47	63	68	141
(b) % of total	74.6	63	68	70.5
(c) Melting point °C	175-6	170-2	170-2	171-2
(d) M.P. when mixed with pure DDS °C	175-6	173-4	173-4	174-5

and alkalized by addition of about 20% NaOH solution. After being chilled on ice the crystals are collected by centrifugation, washed by centrifugation with a minimum volume of ice cold water until neutral to litmus and dried at 60-80°C. The dry residue in the centrifuge tube is dissolved for the most part in a few cc of acetone, centrifuged, the clear solution decanted into a suitable weighing bottle and the operation repeated 2 to 3 times, to separate the acetone-soluble part from the insoluble solid which is discarded. The acetone is allowed to evaporate and the residue dried *in vacuo* to constant weight. The material so obtained from urine, when pure DDS is added to it, had a sharp melting point of 175-176°C. Material so obtained from urine of rabbits given DDS *per os* assayed 100% by a modification of the Bratton and Marshall diazotization technic and usually had a melting point of 170-172°C; when mixed with pure DDS it melted at 173-175°C. Recrystallization of this material by dissolving it in a minimum volume of acetone and slowly adding 5 to 6 volumes of water, centrifugation and drying yielded crystals which had a melting point of 172-174°C. The crystals were decolorized in hot methanol solution

with Norit and recovered by evaporation of the solvent. A final recrystallization from a little methanol yielded DDS melting at 175-176°C alone or mixed with authentic crystals of DDS.

Table I shows the results of fractionation of a sample rabbit urine to which 63 mg of pure DDS had been added as the hydrochloride and similar fractionation of pooled catheterized specimens of urine obtained from 3 rabbits 3 to 6 hours after the oral administration of 0.5 g/kg of DDS in aqueous suspension with gum acacia. In the latter instance the urine was assayed by a modified Bratton and Marshall technic<sup>6</sup> against DDS as a standard and a volume of urine corresponding to the amount of DDS equivalent indicated in the table was used for fractionation.

The data in Table I indicate that at least 63-70% of the total diazotizable material in the urine is unaltered DDS. Parallel assays of the several fractions by diazotization after acid hydrolysis at 100°C and direct diazotization at room temperature gave identical

<sup>6</sup> Smith, M. I., Junge, J. M., and McClosky, W. T., *J. Am. Pharm. Assn., Sci. ed.*, 1948, 37, 461.

values except in fraction one in which the former values were from 10-30% higher, suggesting that a small amount, not over 5% of the total, had been altered to some metabolite, possibly conjugated DDS.

The technic of partition chromatography on filter paper, as originated by Consden, Gordon and Martin<sup>7</sup> has been adapted for the resolution of DDS and several synthetic derivatives from mixtures. Normal butanol saturated with 3% ammonia, benzene saturated with water, and equal parts of benzene and cyclohexane saturated with water were used with ascending columns of narrow ( $\frac{1}{8}$  in. wide) strips of Whatman's No. 1 filter paper. The papers were hung for 3 to 24 hours in a liter graduate cylinder containing 100 cc of one of the above solvents. Colored bands of DDS or monosubstituted derivatives were produced by pipetting the Bratton and Marshal reagents (dissolved in organic solvents to minimize dispersal of the pigments produced) on the dried strips. The p-dimethylaminobenzaldehyde reagent (1% in 3% HCl) of Kühnau,<sup>8</sup> giving a yellow to orange precipitate, has also been found useful. It has been found that rabbit urine containing DDS or certain derivatives, may be chromatographed without prior treatment. Aliquots of 0.01 cc, containing 0.1 to 1.0  $\gamma$  of each sulfone, were used.

Applying such methods to the several fractions in Table I evidence has been obtained to indicate that, in addition to the original urine,

fraction 1 and possibly also fraction 2 contained another compound besides DDS. The original urine and fraction 1 gave 2 distinct bands when chromatographed from the benzene-water solvent. One of these bands, the upper, had the same  $R_F$  value as pure DDS. The other, the lower band ( $R_F < 0.1$ ), was unlike those produced by the monoacetyl derivative of DDS or 3-hydroxy-4,4'-diaminodiphenylsulfone or the sulfide and sulfoxide analogues of DDS.

These results demonstrate that the bulk of the diazotizable material excreted in the urine following the oral administration of DDS to rabbits is unchanged DDS. The procedure described also provides a ready means for determining whether a given derivative of DDS is metabolized to the parent substance or not.

**Summary.** A method is described for the isolation and identification of 4,4'-diaminodiphenylsulfone (DDS) in the urine of rabbits. The method is based on the solubility of the hydrochloride of DDS in water and the relative insolubility of the base in water but its ready solubility in ethyl acetate.

Up to 70% of the total diazotizable material excreted in the urine following oral administration of DDS to rabbits has been isolated as unchanged DDS. Not more than about 5% of the diazotizable material may be in an altered form, and this probably as conjugated DDS. Partition chromatography indicated that it was not the acetylated derivative of DDS.

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<sup>7</sup> Consden, R., Gordon, A. H., and Martin, A. J. P., *Biochem. J.*, 1944, **38**, 224.

<sup>8</sup> Kühnau, W. W., *Klin. Woch.*, 1938, **17**, 116.

## 17067. Fractionation of Serum Cholesterol of Chickens.\*

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In a recent publication<sup>1</sup> we reported that only a small percentage of the total cholesterol was extracted from lyophilized normal human serum by chloroform at about 5°C. The fraction thus extracted was designated as the "readily extractable cholesterol", and extraction was found to be complete in 3 hours. This fraction is very high in the serum of nephrotic patients and hypercholesterolemic rabbits, comprising in some cases apparently all of the serum cholesterol.<sup>1</sup> Since atherosclerosis is a common occurrence in both of these instances, it was felt that further studies on the possible significance of this fraction would be of value.

Various authors<sup>2-5</sup> have shown that spontaneous arteriosclerotic lesions are common in chickens; lesions being evident in some by the time they reach 6 months of age. In the hen these lesions are found in both the elastic and muscular aorta, while they are normally confined to the muscular aorta in the rooster.<sup>6</sup> Since these lesions can develop while the cholesterol concentrations are less than 200 mg per 100 cc of serum,<sup>5</sup> it is evident that the concentration of cholesterol in the plasma can hardly be the chief etiological factor in

the development of the arterial lesions. The purpose of the present investigation was to determine the concentration of the "readily extractable cholesterol" fraction and neutral fat of the serum of old and young chickens with the hope of obtaining some information which might help to explain atherosclerosis in the presence of relatively low concentrations of total serum cholesterol.

*Experimental.* Chickens of both sexes and of 3 age groups were studied. One group was 3 years of age or older, another from about 1 to 2 years and the third from 8-10 weeks old. The diet of the older birds is believed to have been mostly scraps plus some commercial feed. The young chickens received primarily starter mash. In most cases the animals were sacrificed the day they were received in the laboratory, but in a few cases, they were kept in an animal room and fed commercial laying mash for a few days prior to sacrificing. Blood for analyses was obtained by beheading the birds and collecting the blood in a beaker. One-half cc samples of the serum were lyophilized as described previously.<sup>1</sup> The dried serum was extracted with cold chloroform at a temperature of about 5°C for definite periods of time. The bottles were shaken every 10 to 15 minutes for the first 3 hours and then occasionally for the remainder of the extraction period. One or 2 bottles were filtered in the cold at different periods of time and the cholesterol content of the extract determined. The results shown in Tables I and II are typical of those obtained. For comparison, a number of results obtained on normal human sera are shown in Table III. The fraction designated as "neutral fat plus cholesterol" consists of neutral fat, cholesterol and cholesterol esters. The method employed for its determination has been described previously.<sup>1</sup>

*Discussion.* It will be seen from the experimental results that the "readily extractable" fraction is quite high in the serum of old hens; the older hens behaving in this re-

\* This investigation was made possible through the financial support of the Charles C. Haskell & Co., Richmond, Va., to whom we are greatly indebted.

<sup>1</sup> Forbes, J. C., Dillard, G. H. L., Porter, W. B., and Petterson, Olga, *Proc. Soc. Exp. Biol. and Med.*, 1948, **68**, 240.

<sup>2</sup> Fox, H., *Bull. N. Y. Acad. Med.*, 1939, **15**, 748.

<sup>3</sup> Horlick, L., Dauber, D., and Katz, L. N., *Am. Heart J.*, 1948, **35**, 863.

<sup>4</sup> Keston, H. D., Meaker, D. R., and Jobling, J. W., *Proc. Soc. Exp. Biol. and Med.*, 1936, **34**, 818.

<sup>5</sup> Horlick, L., and Katz, L. W., *J. Lab. Clin. Med.*, 1948, **33**, 733.

<sup>6</sup> Dauber, D. V., *Arch. Path.*, 1944, **38**, 46.

<sup>7</sup> Dauber, D. V., and Katz, L. N., *Arch. Path.*, 1943, **36**, 473.

<sup>8</sup> Fox, H., in Cowdry, E. V.: *Arteriosclerosis*, New York, The Macmillan Co., 1933, Chap. 6.

TABLE I.  
Extraction of Cholesterol from Lyophilized Serum of Female Chickens.

Number	Age	Cholesterol extracted in			Total Chol., mg %	Neutral fat + Chol., mg %
		3 hr, mg %	24 hr, mg %	72 hr, mg %		
1	>3 yrs.	225	222	—	234	1248
2	" "	152	168	—	162	1207
3	" "	158	171	—	169	1355
4	About 3 yrs.	200	196	—	216	2259
5	" "	115	126	—	137	1487
6	" "	83	82	—	115	847
7	" "	34	71	—	275	419
8	About 2 yrs.	123	136	—	183	969
9	" "	17	18	—	207	309
10	" "	4	5	—	120	226
11	About 10 wks.	8	12	18	129	773
12	" "	7	11	17	132	333
13	" "	16	100	110	129	232
14	" "	11	8	11	132	387

TABLE II.  
Extraction of Cholesterol from Lyophilized Serum of Male Chickens.

Number	Age	Cholesterol extracted in			Total Chol., mg %	Neutral fat + Chol., mg %
		3 hr, mg %	24 hr, mg %	72 hr, mg %		
1	>3 yrs.	7	9	9	117	234
2	" "	11	13	17	136	281
3	" "	13	67	126	138	235
4	" "	8	7	6	128	310
5	About 3 yrs.	7	8	10	145	202
6	About 2 yrs.	7	9	12	94	250
7	" "	17	72	145	202	437
8	About 1 yr.	7	6	7	139	303
9	" "	8	6	12	119	249
10	About 10 wks.	9	11	28	144	258
11	" "	16	16	32	147	258
12	" "	15	110	107	141	303
13	" "	8	12	18	129	773
14	" "	11	11	65	113	387

gard very similar to the nephrotic patient, or the hypercholesterolemic rabbit.<sup>1</sup> Roosters, both old and young, as well as young female chickens, showed a low value for this fraction, but in some cases the amount extracted increased with increased extraction time. We have obtained a similar increased extraction with increased extraction time in a few apparently normal human subjects as well as in a considerable number of patients. The reason for this deviation from the average normal is unknown, but preliminary experimental work with roosters indicates that it is probably dependent upon dietary composition.

It will be noted that old hens showed, on the whole, a much higher level of serum fat

than did old roosters or young chickens. There also appears to be some correlation between the percentage of the cholesterol present in the "readily extractable" form and the neutral fat content of the serum, especially in the case of the old hens. Dauber and Katz<sup>7</sup> have emphasized the close similarity between the lesions produced by the administration of cholesterol to roosters and the spontaneous lesions in old birds, described by Fox.<sup>8</sup> They also have pointed out their dissimilarity from those which usually occur in roosters. The possible significance of the concentration of the "readily extractable" cholesterol as an etiological factor in the development of the lesions found in old hens and cholesterol-fed

TABLE III.  
Extraction of Cholesterol of Lyophilized Normal Human Serum.

Subject	Cholesterol extracted in				Total Chol., mg %	Neutral fat + Chol., mg %
	3 hr, mg %	24 hr, mg %	27 hr, mg %	96 hr, mg %		
A.L.F.	20	20	25	—	227	428
D.F.M.	30	31	35	—	322	511
C.J.R.	28	25	27	—	270	428
A.F.	22	22	20	—	226	404
D.F.	22	29	28	—	240	594
A.B.	30	22	28	—	286	392
K.N.	20	26	21	23	215	333
G.W.J.	22	27	27	38	272	551
R.B.	34	38	37	35	272	735
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L.S.	36	76	102	—	233	722
M.D.	24	24	32	—	210	365

chickens is being investigated.

Although the data which have been presented are not sufficient to enable one to draw conclusions as to the physical state in which cholesterol exists in serum, they do indicate that it probably exists in at least three states not related to the usual classification of free and ester cholesterol. These are (a) a fraction not extracted by chloroform under the conditions used; (b) a fraction which is readily extractable by chloroform, a 3 hour extraction period being adequate for its complete extraction; and (c) a fraction which is slowly extractable. We believe that (a) possibly represents cholesterol which is held firmly by the serum proteins, while (b) possibly represents cholesterol which is free in the serum or which is associated with other

lipid materials such as fats and phospholipids. Our experimental evidence indicates that all the fractions contain both free and ester cholesterol.

*Summary.* A large percentage of the cholesterol of lyophilized serum from old hens is present in the "readily extractable" fraction. The concentration of this fraction in the serum of roosters and young chickens is quite low but, in some cases, the amount extracted increases with increased extraction time. There appears to be a correlation between the percentage of the total cholesterol present in the "readily extractable" fraction and the neutral fat content of the serum of old hens.

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## 17068. Acetaldehyde Disappearance and Acetoin Formation *in vitro*.\*

W. W. WESTERFELD.

From Department of Biochemistry, Syracuse University College of Medicine, Syracuse, N. Y.

The presence of a diphosphothiamin enzyme in animal tissues capable of forming acetoin from pyruvate, and the utilization of acetaldehyde as a substrate in this reaction, has been described.<sup>1</sup> A limited study of this reaction,

in brain homogenate was reported previously.<sup>2</sup> The present study was devoted to a comparison of the abilities of various tissues to meta-

<sup>1</sup> Green, D. E., Westerfeld, W. W., Vennesland, B., and Knox, W. E., *J. Biol. Chem.*, 1942, 145, 69.

<sup>2</sup> Stotz, E., Westerfeld, W. W., and Berg, R. L., *J. Biol. Chem.*, 1944, 152, 41.

\* Aided by a grant from the Nutrition Foundation, Inc.

TABLE I.  
 Acetaldehyde Disappearance and Acetoin Formation *in Vitro* with Rat Tissues.

Substrate	Micromols added	Brain		Leg muscle		Liver	
		AcH disapp.,	Acetoin formed,	AcH disapp.,	Acetoin formed,	AcH disapp.,	Acetoin formed,
		$\gamma$	$\gamma$	$\gamma$	$\gamma$	$\gamma$	$\gamma$
None	—	71*	36*	71	69	144	21
Pyruvate	3	95*	95*	124	164	142	37
Glucose	6	92†	100†	68	76	144	23
$\alpha$ -Ketoglutarate	6	89	67	125	124	140	0
Lactate	12	73	58	64	73	142	0
Alanine	12	75	58	69	71	141	0
Phosphoglycerate	6	70	50	96	107	143	0
Citrate	6-100	71	28	47	37	147	0

\* Approximately the same results were obtained in oxygen and nitrogen.

† Approximately the same results were obtained in oxygen; in nitrogen the acetaldehyde disappearance was similar but the acetoin formation was only 46  $\gamma$ .

The reproducibility of the results is indicated by the following data for 11 experiments with rat brain in the absence of substrate. Acetaldehyde disappearance: mean = 71, S.D. = 10.3, S.E. = 3.1, range = 57-88. Acetoin formed: mean = 36, S.D. = 3.55, S.E. = 1.07, range = 31-42. The effect of each substrate was compared with a simultaneous control in 2 or more experiments.

bolize acetaldehyde and form acetoin, and to the effect of added substrates upon these reactions.

**Methods.** To a Warburg vessel were added 1 cc of tissue homogenate in saline containing 250 mg of fresh tissue, 1.5 cc of 0.1 M phosphate buffer, pH 7.4, 20  $\gamma$  diphosphothiamine in 0.1 cc water, 0.2 cc of water or substrate solution, and 0.3 cc of 0.05% acetaldehyde in the side arm. After 10 minutes equilibration at 37.5°C, the acetaldehyde was tipped in and shaken for 1 hour. A tungstic acid filtrate of the contents of the cooled flask was then prepared and analyzed for the acetaldehyde remaining<sup>3</sup> and the acetoin formed.<sup>4</sup> Mechanical and volatilization losses were small in the absence of tissue.

**Results.** Some of the results with rat tissues are shown in Table I. Brain and muscle breis removed acetaldehyde at about the same rate, but muscle formed twice as much acetoin as brain. Kidney brei in the absence of added substrate was similar to brain. Liver metabolized acetaldehyde at a much faster rate, but formed little acetoin. Corresponding results were previously obtained *in vivo*<sup>5</sup> where large amounts of acetoin were

formed from acetaldehyde only when the dominant role of the liver in acetaldehyde metabolism was eliminated by hepatectomy.

The addition of pyruvate, or other substrates capable of forming pyruvate in the brei, increased both the acetaldehyde disappearance and the acetoin formation. Pyruvate and  $\alpha$ -ketoglutarate gave large increases with both brain and muscle; glucose was also very effective in brain, but not muscle, while phosphoglycerate stimulated the reaction somewhat more in muscle than in brain. In liver, acetoin formation disappeared completely in the presence of most substrates.

Other results obtained with 6-12 micromols of substrate added to brain were: significant increases (30-60%) in acetoin formation from glycogen, lactate, alanine, phosphoglycerate and malate; minor increases (10-25%) from succinate, fumarate, glucose-1-phosphate, fructose and glycerol phosphate; no effect from butyrate, acetate, propionate, citrate, glutamate, cis-aconitate, galactose and glycerol. Anesthetization of the rat with nembutal or the addition of nembutal *in vitro* did not affect the reaction in brain tissue. The addition of 0.5 mg methylene blue to brain tissue increased the acetaldehyde disappearance 50% and the acetoin formation by 150%. When added together with pyruvate or glucose, methylene blue increased both the acetaldehyde disappearance and acetoin formation be-

<sup>3</sup> Stotz, E., *J. Biol. Chem.*, 1943, **148**, 585.

<sup>4</sup> Westerfeld, W. W., *J. Biol. Chem.*, 1945, **161**, 495.

<sup>5</sup> Lubin, M., and Westerfeld, W. W., *J. Biol. Chem.*, 1945, **161**, 503.



TABLE III.  
Extraction of Cholesterol of Lyophilized Normal Human Serum.

Subject	Cholesterol extracted in				Total Chol., mg %	Neutral fat + Chol., mg %
	3 hr, mg %	24 hr, mg %	27 hr, mg %	96 hr, mg %		
A.L.F.	20	20	25	—	227	428
D.F.M.	30	31	35	—	322	511
C.J.R.	28	25	27	—	270	428
A.F.	22	22	20	—	226	404
D.F.	22	29	28	—	240	594
A.B.	30	22	28	—	286	392
K.N.	20	26	21	23	215	533
G.W.J.	22	27	27	38	272	551
R.B.	34	38	37	35	272	735
G.C.	37	37	42	43	331	705
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W. W. WESTERFELD.

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The presence of a diphosphothiamin enzyme in animal tissues capable of forming acetoin from pyruvate, and the utilization of acetaldehyde as a substrate in this reaction, has been described.<sup>1</sup> A limited study of this reaction,

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in brain homogenate was reported previously.<sup>2</sup> The present study was devoted to a comparison of the abilities of various tissues to meta-

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<sup>2</sup> Stotz, E., Westerfeld, W. W., and Berg, R. L., *J. Biol. Chem.*, 1944, 152, 41.

penicillin. L type cultures were not observed in any of these plates. However, from one earth sample a large Gram positive bacillus was isolated which, when transplanted on penicillin plates, produced pleomorphic forms and tiny L type colonies. Neither the first nor the second Gram positive bacillus produced spores.

These Gram positive bacilli probably originated from the soil in which pleuropneumonia-like organisms are often present. Thus a contamination of the cultures with such organisms must be considered. Usually it is difficult to exclude this possibility and to prove that the L form grow from the bacilli. The needed proof was obtained with a third Gram positive bacillus. This bacillus produced spores and the L form grew in the same manner from the heated spores as from the original culture. This observation, while it excludes an accidental contamination of the cultures, does not prove in itself that the L cultures represent a changed growth form of the bacillus. They could be parasites of the bacilli and Klieneberger,<sup>1</sup> for example, even at present prefers this hypothesis. In the opinion of the author the evidence in the case of Gram negative bacilli<sup>2-4</sup> supports the conception that the L forms and the bacilli are growth forms of the same organism.

The third Gram positive rod was obtained from a thioglycollate broth inoculated from a wound. Growth in the first subculture developed only anaerobically, and on further subcultures also anaerobic growth was more abundant than aerobic. The colonies on blood agar plate incubated aerobically were flat and slightly irregular, and after a few days the culture spread as a thin film on the surface of the medium. Large elongated spores, usually at the ends of the rods, developed abundantly. A distinguishing property of the bacillus was its tendency to swell into large pleomorphic forms. Some of the bacterial threads spread-

ing on blood agar plate autolyzed and disintegrated and the fragments swelled into small and large round forms. In thioglycollate broth some of the rods grew to large size and became markedly swollen around the developing spores. This tendency to form round bodies is rarely observed in Gram positive rods. Exact identification of saprophytic strains is difficult and has not been attempted. However, the development of spreading growth on the surface of the agar under aerobic conditions and the formation of acid without gas in various sugars makes it probable that it belongs to the genus *Bacillus*.

A thioglycollate broth culture of this strain was planted on soft agar plates containing 10% horse serum and 400 units of penicillin per cc. The plates were made anaerobic using Fortner's technic.<sup>5</sup> Use of a similar medium permitted isolation of L type colonies from various Gram negative bacilli.<sup>3,4</sup> The development of the third strain on this medium was similar to that of Gram negative bacilli which produced L type colonies. Many bacilli swelled within a few hours into large flat round bodies. From these tiny colonies developed which grew best in transplants on soft nutrient agar plates without serum. The tiny colonies on the original penicillin plates and in the subcultures are illustrated in the photographs. Their appearance and morphology were similar to the L<sub>1</sub> and to the other L type colonies obtained from Gram negative bacilli. The colonies grew into the medium and consisted of soft fragile cells which were often very small and had the tendency to swell into large bodies.

In order to determine whether L cultures can be obtained from heated spores, well-sporulated broth cultures were sealed in thin glass tubes and immersed in water at 85°C for 10 minutes. (Previous experiments had shown that the spores were killed in boiling water). The heated spores when transferred to penicillin plates produced no growth. Broth cultures of bacilli obtained from the heated spores produced L type colonies on penicillin plates as the original cultures.

<sup>1</sup> Klieneberger-Nobel, E., *J. Hygiene*, 1948, 45, 407.

<sup>2</sup> Dienes, L., *Cold Spring Harbor Symposia Quant. Biol.*, 1947, 11, 51.

<sup>3</sup> Dienes, L., *J. Bacteriology*, 1948, 56, 445.

<sup>4</sup> Dienes, L., *J. Bacteriology*, 1949, in press.

<sup>5</sup> Dienes, L., and Smith, W. E., *J. Bacteriology*, 1944, 48, 125.

yond that obtained with either methylene blue or the substrate alone.

It is reasonable to suppose that the increased acetoin formation in the presence of added substrate represented an increased amount of pyruvate available for condensation with the excess acetaldehyde. The ineffectiveness of other members of the citric acid cycle, as compared with  $\alpha$ -ketoglutarate, the effect of glucose in brain as compared with muscle, and the comparison of glucose with fructose or glucose-1-phosphate are noteworthy considerations in any interpretation of these results.

Further studies on the disappearance of acetaldehyde in liver brei were made with 0.3 cc of 0.15% acetaldehyde in the side arm. Rat liver removed about 200  $\gamma$  acetaldehyde during the incubation; added glucose had no effect. Small increases (10%) in the amount removed resulted from the addition of pyruvate, lactate or alanine. 100  $\mu$ M of citrate or 0.5 mg methylene blue increased the acetalde-

hyde disappearance by 25% and 40%. Dog liver gave similar results except that methylene blue had no effect. The methylene blue effect in rat liver as contrasted with dog liver is possibly related to the presence of xanthine oxidase in rat liver only; methylene blue stimulates aerobic oxidation by xanthine oxidase.

*Summary.* In agreement with previous *in vivo* studies, liver homogenate metabolized acetaldehyde more rapidly than other tissues and without the formation of much acetoin. Methylene blue stimulated the aerobic metabolism of acetaldehyde in rat liver but not dog liver.

Skeletal muscle formed more acetoin from acetaldehyde than did brain or kidney. Pyruvate,  $\alpha$ -ketoglutarate and phosphoglycerate increased the acetoin formation from acetaldehyde by muscle; pyruvate,  $\alpha$ -ketoglutarate and glucose were effective stimulators of acetoin formation in brain.

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### 17069. Isolation of an L Type Culture from a Gram Positive Spore-Bearing Bacillus.\*

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During the last 10 years L type cultures have been isolated from many Gram negative bacilli. The author tried repeatedly to apply to Gram positive bacilli the methods which proved successful in Gram negative species. Success was obtained first in the summer of 1948 with a Gram positive bacillus isolated from a contaminated blood culture. The broth cultures of this bacillus were pleomorphic. After 24 - 48 hours of incubation almost all bacilli swelled to round bodies as in the cul-

tures of some Bacteroides strains. This pleomorphic culture transferred to media of various compositions produced only bacterial growth, but when a penicillin cup was placed on soft horse-serum agar plates and the plates were incubated anaerobically, a few microscopical L type colonies developed near the edge of the zone of inhibition. Growth of these tiny colonies in pure culture was obtained by cutting out a block of agar containing the colonies and transferring it to horse serum agar containing 400 units of penicillin per cc. The colonies grew without difficulty in subsequent subcultures.

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Following this observation several samples of dust and earth were planted on similar plates containing varying concentrations of

L colonies grew easier in subculture. Among the descendants of the colony which was least susceptible to the influence of penicillin, only a few bacilli swelled to large form when their growth was inhibited by penicillin and only a few L type colonies were produced. A similar individual behavior of different strains belonging to the same species has been noted in all species which produce L colonies, but the differences are usually apparent only between strains of different isolation.

The L type cultures obtained from Gram positive bacilli differ from each other and from those obtained from Gram negative bacilli in many respects, although their fundamental morphology and structure of the colonies conform to those of the pleuropneumonia group. The colonies obtained from the two bacilli mentioned first remained very small ( $10-20\ \mu$ ) on the original plates and in the early transfers stained poorly with methylene blue. After several transfers they grew more abundantly. They grew equally well with or without horse serum in the medium. The hardness of the agar played an important role in their growth. In the absence of horse serum growth developed only on hard agar plates containing 1.5%-2% of agar. If the agar was softened by the addition of equal amounts of broth, it did not support their growth. Soluble starch markedly improved growth, while glucose, maltose, sucrose and penicillin exerted no influence. The colonies continued to increase in size for a period of two weeks. No growth could be obtained in nutrient or thioglycollate broth, although the colonies transferred with a block of agar into the broth continued to increase in size on the agar. In morphology these cultures were quite similar to the filterable strains isolated from sewage by Laidlow and Elford.<sup>6</sup> The cultures

consist of very small ( $0.3-0.5\ \mu$ ), fragile coccoid and bacillary forms and they swelled only to moderate size on the surface of the colonies. The strains of Laidlow and Elford grow abundantly in broth and aerobically on nutrient agar. In this respect they differ considerably from ours.

Young colonies of the L strain isolated from the third spore-bearing bacillus were more similar in appearance to the L cultures isolated from *Streptobacillus moniliformis* and *Bacteroides* and consisted of similar organisms. If the colonies were well spaced, they increased in size for several weeks extending as an opaque mass below the surface of the agar reaching the size of 1-2 mm. They grew best on nutrient agar in the absence of horse serum. In contrast to the other two strains growth developed only on soft agar plates and was inhibited by the usual concentration of agar. Sucrose seemed to favor their growth but its effect was not marked; starch exerted no influence. No growth was obtained in nutrient or thioglycollate broth. The original bacillary forms have not been recovered thus far from any of the three L cultures isolated from Gram positive bacilli.

*Summary.* Colonies similar in appearance and properties to the L type colonies of Gram negative bacilli were isolated from cultures of several large Gram positive bacilli. The L type colonies obtained from a spore-producing strain grew equally well from cultures obtained from heated spores as from the original culture, indicating that the L type colonies grew from the bacilli and were not an accidental contamination of the culture.

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<sup>6</sup> Laidlow, P. P., and Elford, W. J., *Proc. Roy. Soc. London B.*, 1936, **120**, 292.

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FIG. 1.

1. Bacilli from a broth culture. Slightly stained with crystal violet. ( $\times 2000$ .)
2. Culture with spreading growth on aerobic blood agar plate. ( $\times 2$ .)
3. Tiny colonies on penicillin plate after 5 days' incubation. ( $\times 2$ .)
4. Tiny colonies from a subculture on a soft nutrient agar plate. Unstained. ( $\times 100$ .)
5. A colony similar to those in Photograph 4 stained with methylene blue on the surface of the agar. ( $\times 100$ .)
6. Large bodies in the periphery of a tiny colony stained with methylene blue. ( $\times 2000$ .)

It was interesting to observe the development of the cultures when a soft agar plate was inoculated with heated spores and 100-500 units of penicillin was deposited in a small trough made in the agar. The plates were incubated anaerobically. No growth developed in a wide area around the penicillin trough. The penicillin diffused in this area before the spores germinated. Many tiny colonies developed in the adjoining zone. Here the spores germinated and the young vegetative cells were exposed to the diffusing penicillin. The tiny colonies consisted of large round forms produced by swelling of the vegetative cells and many L colonies began to grow from these large forms. Further away from the penicillin trough the usual bacillary colonies developed. The observations reported indicate

that L forms were not cultivated directly from the heated cultures. They apparently survive with the bacteria and develop under appropriate conditions from the vegetative cells. The L strains isolated from the second and third bacilli were tested for heat resistance. Heating of the cultures for 15 minutes at  $60^{\circ}\text{C}$  destroyed their viability.

It is of interest also that descendants of single bacillary colonies varied considerably in their tendency to produce L type colonies and in the viability of these colonies. In one experiment 4 colonies were picked from a culture of bacillus No. 3 on a blood agar plate. Marked differences persisting in subculture were present among the descendants of these colonies. The descendant of one produced more L type colonies than the others and the

Comparable groups of chicks having hemoglobin values ranging from 2 to 4 g % were then arranged. Experimental treatments were followed by determining hemoglobin levels at 2 to 4 day intervals, using the alkaline oxy-hemoglobin method<sup>8</sup> as adapted for the Evelyn colorimeter.<sup>9</sup> Blood samples were taken directly by puncture of a wing vein.

Dosages of phenylhydrazine hydrochloride from 1 to 6 mg per 100 g body weight were tested. 2.0 mg was selected as the smallest dosage which caused a suitable decrease in hemoglobin level within 2 days. It was also observed that a rapid return to normal hemoglobin level occurred if the ration contained adequate folic acid. The hemoglobin level of normal chicks dropped to about 5 g % within 2 days after the injection of phenylhydrazine hydrochloride. Three to 4 days following this anemic condition, their hemoglobin level had returned to normal. Consequently, small amounts of folic acid must be given to the folic acid depleted, anemic birds to prolong the period of recovery so that differences in rate of hemoglobin regeneration can be measured.

**Experiment 1.** Four groups of deficient chicks, having hemoglobin values of 2 to 4 g %, were selected on the 16th day (2 days following the injection of 2.0 mg of phenylhydrazine hydrochloride per 100 g body weight). Six chicks were used in each group and no losses occurred during the following treatments (1) control group, untreated, (2) liver extract (Lilly, reticulogen, 20 U.S.P. units per cc), 1 unit per bird, injected, (3) folic acid, 20  $\gamma$  per bird, injected, (4) liver extract, 1 unit per bird and folic acid, 20  $\gamma$  per bird, injected. The dosages were given on the 16th, 18th and 20th days.

The changes in the hemoglobin values following the above treatments are shown in Fig. 1. Liver extract alone did not influence the rate of hemoglobin regeneration. By the 24th day, the hemoglobin values of the groups receiving folic acid and folic acid in combination with liver extract had returned to

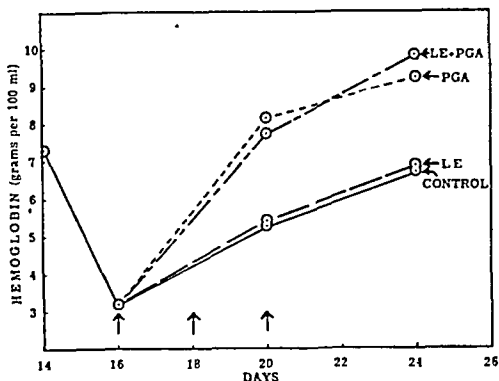


FIG. 1.

Rate of Hemoglobin Regeneration in Anemic Chicks.

The control group was untreated. In the other groups each bird received: LE—1 unit liver extract; PGA—20  $\gamma$  folic acid; LE + PGA—1 unit liver extract and 20  $\gamma$  folic acid, injected on the 16th, 18th, and 20th days.

normal and did not differ significantly from each other. The least significant difference between treatments on the 24th day was 1.23 g %.

**Exp. 2** The pattern of Exp. 2 was similar to that used in Exp. 1 except that the depletion period was extended to 21 days before injection of phenylhydrazine hydrochloride. Pteroylheptaglutamic acid and pteroyltriglutamic acid were tested at equimolar dosage to pteroylglutamic acid (PGA). All of the 5 chicks used in each test group survived.

As shown in Fig. 2, liver extract alone did not increase the rate of hemoglobin regeneration. PGA alone did. The triglutamate and heptaglutamate forms were as active as PGA. Liver extract with folic acid resulted in more rapid regeneration of hemoglobin than was observed on folic acid alone. A significant difference in the average hemoglobin values of these two groups was noted on the 29th day and again on the 31st and 33rd days. The least significant difference between treatments by the 33rd day was 1.27 g %.

**Exp. 3.** Chicks were depleted for 24 days on a folic acid deficient, purified ration in which sucrose was used as the source of carbohydrate. In addition to the treatments used in Exp. 1 and 2, pure vitamin B<sub>12</sub> was tested alone and in combination with folic acid. In the control group, 5 of the 8 birds

<sup>8</sup> Drabkin, D. L., and Austin, J. H., *J. Biol. Chem.*, 1932, **98**, 719.

<sup>9</sup> Evelyn, K. A., *J. Biol. Chem.*, 1936, **115**, 63.

# 17070. Effect of Folic Acid, Liver Extract, and Vitamin B<sub>12</sub> on Hemoglobin Regeneration in Chicks.\*

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No change in the blood hemoglobin level has been found to accompany the growth response of chicks to the injection of antipernicious anemia (APA) liver extracts<sup>1</sup> or vitamin B<sub>12</sub><sup>2</sup> when fed a corn-soybean oil meal basal ration containing adequate folic acid. The importance of folic acid for hemoglobin regeneration has been demonstrated with experimental anemias in several different species showing anemia and in the therapy of human macrocytic anemias. APA liver extracts have been found to be ineffective for the cure of anemia induced by folic acid deficiency in the chick<sup>3,4</sup> and in the rat.<sup>5</sup> Data presented here, based upon the rate of hemoglobin regeneration after production of a severe, chemically-induced anemia in folic acid depleted chicks, show that liver extracts or vitamin B<sub>12</sub> are ineffective in increasing the rate of hemo-

globin formation when given alone, but show a stimulatory effect when given with folic acid.

**Experimental.** Straight-run (New Hampshire ♂♂ x Single Comb White Leghorn ♀♀) crossbred chicks which were the progeny of hens fed diet B-1 described previously<sup>6</sup> were used in all studies. The chicks were housed in electrically heated batteries with raised screen floors. Feed and water were supplied *ad libitum*. The chicks were wing-banded when one day old. Weights were recorded at weekly intervals during the depletion period.

All chicks received a folic acid deficient purified ration containing dextrin 61, alcohol-extracted casein 18, gelatin 10, salts V 6,<sup>7</sup> soy oil 5, 1-cystine 0.3, and fortified haliver oil (60,000 U.S.P. units of vitamin A, 6,000 A.O.A.C. units of vitamin D<sub>3</sub> per g) .04 g; thiamin 0.3, riboflavin 0.6, nicotinic acid 5.0, pyridoxine 0.4, calcium pantothenate 2.0, biotin 0.02, choline chloride 150, inositol 100, 2-methyl-1,4-naphthoquinone 0.05 and  $\alpha$ -tocopherol 0.3 mg.

Chicks fed the folic acid deficient, purified ration developed a slight anemia in 14 to 24 days. Hemoglobin levels varied widely, but the decrease averaged 1 to 2 g below normal. At the end of this depletion period a single intramuscular injection of phenylhydrazine hydrochloride (2.0 mg per 100 g body weight) was given. Severe anemia developed within 2 days (Hb range, 1-5 g %). Reagent grade phenylhydrazine hydrochloride was treated with charcoal and recrystallized from hydrochloric acid solution. An aqueous solution (10 mg per cc) was injected into the pectoral muscle using a 1 cc calibrated hypodermic syringe.

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<sup>1</sup> Nichol, C. A., Robblee, A. R., Cravens, W. W., and Elvehjem, C. A., *J. Biol. Chem.*, 1947, **170**, 419.

<sup>2</sup> Nichol, C. A., Dietrich, L. S., Cravens, W. W., and Elvehjem, C. A., *Proc. Soc. Exp. Biol. and Med.*, 1949, **70**, 40.

<sup>3</sup> O'Dell, B. L., and Hogan, A. G., *J. Biol. Chem.*, 1943, **149**, 323.

<sup>4</sup> Stokstad, E. L. R., and Jukes, T. H., *Proc. Soc. Exp. Biol. and Med.*, 1946, **62**, 112.

<sup>5</sup> Kodicek, E., and Carpenter, K. J., *Biochem. J.*, 1948, **43**, *Proc. Biochem. Soc.*, 1.

<sup>6</sup> Robblee, A. R., Nichol, C. A., Cravens, W. W., Elvehjem, C. A., and Halpin, J. G., *Poultry Sci.*, 1948, **27**, 442.

<sup>7</sup> Briggs, G. M., Jr., Luckey, T. D., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1943, **148**, 163.

of hemoglobin regeneration following treatment with liver extract and folic acid was significantly greater than the rate observed following treatment with an equal dosage of folic acid alone. Less than 1  $\gamma$  of folic acid was present in the 1 U.S.P. unit of liver extract injected. The component causing the stimulation of hemoglobin regeneration in the presence of folic acid appears to be vitamin B<sub>12</sub>.

The metabolic interrelationship between folic acid and vitamin B<sub>12</sub> is still obscure. Since in the anemic chicks, folic acid alone in large amounts will bring about rapid and complete return to normal hemoglobin level, the observed stimulation of hemoglobin regeneration may be due to an indirect action of vitamin B<sub>12</sub> which makes more folic acid available to the animal organism. Vitamin B<sub>12</sub> may also act directly in the normal mechanism of hemoglobin formation. This is implied by the activity of vitamin B<sub>12</sub> in cases of pernicious anemia. The chicks used in these studies, like those used for the growth assay of vitamin B<sub>12</sub>,<sup>2</sup> were depleted by control of the hen's diet. Under the special conditions described, the stimulation of hemoglobin regeneration may be a demonstration of the direct role of vitamin B<sub>12</sub> in the mechanism of hemoglobin formation. This work emphasizes that folic acid and vitamin B<sub>12</sub> may have distinct and separate functions and indicates that vitamin B<sub>12</sub> does not have optimum activity unless folic acid is present in adequate amounts.

The effectiveness of the technic is demonstrated by the similar response to the three forms of folic acid shown in Fig. 2. In each experiment, analysis of variance was applied on the last day. This is a more severe test of significance, since individual variation becomes greater as the interval following treatment increases. For example, in experiment 2 the least significant difference between treatments on the 31st day was 1.05 g % hemoglobin. By the 33rd day the least sig-

nificant difference had increased to 1.27 g %.

Phenylhydrazine hydrochloride is used in the therapy of polycythemia vera. The anemia induced is due to hemolysis of the mature red blood cells, but the mechanism is not known.<sup>10</sup> Yeshoda and Damodaran<sup>11</sup> used a phenylhydrazine induced anemia in rats in a study of the influence of tryptophan on hemoglobin formation. The application of this technic to folic acid depleted chicks increases the range over which differences in hemoglobin levels can be measured and permits selection within narrow limits of comparable groups of chicks having a severe anemia.

**Summary.** A severe anemia was induced in chicks by the intramuscular injection of phenylhydrazine hydrochloride (2.0 mg per 100 g body weight) following a depletion period on a folic acid deficient purified ration. Chicks were selected within narrow limits (2-4 g % Hb) and arranged into comparable groups to study the effect of folic acid, liver extract and vitamin B<sub>12</sub> on the rate of hemoglobin regeneration.

Liver extract alone did not influence the rate of hemoglobin formation. When the depletion period was 21 days or longer, the combination of liver extract and folic acid caused a more rapid regeneration of hemoglobin than treatment with a similar dosage of folic acid alone. Pure vitamin B<sub>12</sub> completely replaced liver extract in stimulating the formation of hemoglobin in the presence of folic acid.

The authors express appreciation to Dr. W. W. Cravens, Poultry Department, University of Wisconsin, and Dr. A. R. Robblee, Poultry Department, University of Alberta, for their cooperation.

<sup>10</sup> Sollmann, T., *A Manual of Pharmacology*, W. B. Saunders Co., Philadelphia and London, 1942, 6th ed., 666.

<sup>11</sup> Yeshoda, K. M., and Damodaran, M., *Biochem J.*, 1947, **41**, 382.



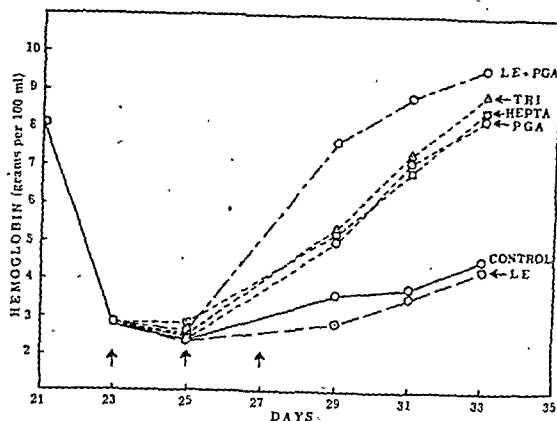


FIG. 2.

## Rate of Hemoglobin Regeneration in Anemic Chicks.

The control group was untreated. In the other groups each bird received: LE—1 unit liver extract injected on the 23rd, 25th, and 27th days; HEPTA—pteroylheptaglutamic acid and TRI—pteroyltriglutamic acid at equimolar dosage to PGA—pteroylglutamic acid, 5  $\gamma$ , 10  $\gamma$ , and 20  $\gamma$  injected on the 23rd, 25th, and 27th days respectively; LE + PGA—1 unit liver extract and 5  $\gamma$  folic acid injected on the 23rd day, 1 unit liver extract and 10  $\gamma$  folic acid injected on the 25th day, 1 unit liver extract and 20  $\gamma$  folic acid injected on the 27th day.

survived on the 38th day and 3 survived beyond the 42nd day. At least 7 of the 8 chicks survived beyond the 42nd day in each of the other test groups.

The average hemoglobin values for each group are shown in Fig. 3. The pattern of hemoglobin regeneration induced by vitamin B<sub>12</sub> was the same as that observed with liver extract. Vitamin B<sub>12</sub> alone had no greater effect than liver extract, yet in combination with folic acid the rate of hemoglobin regeneration was more rapid than that resulting from treatment with folic acid alone. By the 42nd day, the least significant difference between treatments was 1.20 g % hemoglobin.

In addition to the group shown in Fig. 3 which received 1  $\gamma$  of vitamin B<sub>12</sub> plus 10  $\gamma$  folic acid on the 26th and 29th days, a second group of 8 chicks which received 0.1  $\gamma$  of vitamin B<sub>12</sub> plus 10  $\gamma$  of folic acid on these days showed an average hemoglobin value of 8.24 g % on the 42nd day and another group which received double the dosage of folic acid alone (20  $\gamma$ ) on these days had an average hemoglobin value of 7.71 g % on the 42nd day.

*Discussion.* A series of 5 experiments in

all were carried out, each of which included the 4 experimental treatments outlined in Exp. 1. Liver extracts used in the treatment of pernicious anemia were not effective in stimulating the rate of hemoglobin regeneration in anemic chicks depleted of folic acid. In each of the 4 experiments having a depletion period of 21 days or longer, the rapid rate

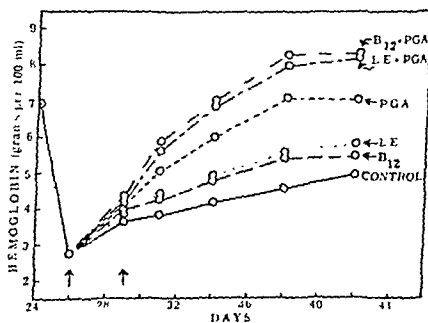


FIG. 3.

## Rate of Hemoglobin Regeneration in Anemic Chicks.

The control group was untreated. In the other groups each bird received: LE—1 unit liver extract; B<sub>12</sub>—1  $\gamma$  vitamin B<sub>12</sub>; PGA—10  $\gamma$  folic acid; LE + PGA—1 unit liver extract and 10  $\gamma$  folic acid; B<sub>12</sub> + PGA—1  $\gamma$  vitamin B<sub>12</sub> and 10  $\gamma$  folic acid, injected on the 26th and 29th days.

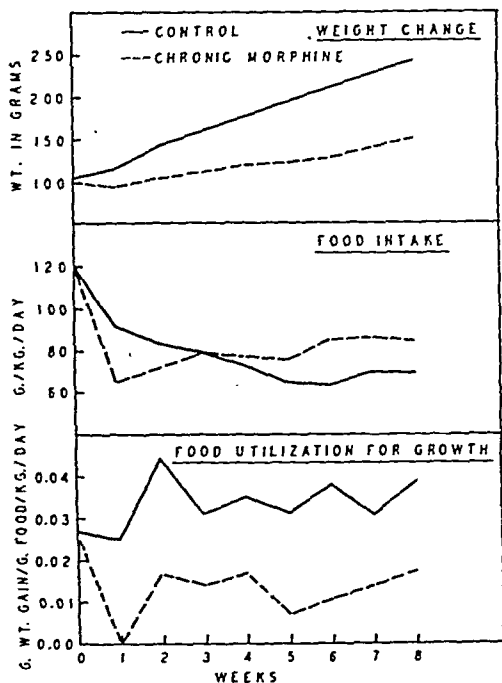


FIG. 1.

The effect of chronic morphine poisoning on the growth, food intake, and food utilization of the albino rat.

weight, food intake in g/kg and grams of weight gain per gram of food consumed per kilo of body weight per day were about equal in the two groups. (Fig. 1). This latter figure gives some indication of the utilization of food for synthesis of body tissues. During the period of the experiment, the rats receiving morphine showed a slight loss in weight during the first week followed by a gradual increase during the ensuing seven weeks. The rate of growth was much slower than in the control group. This great difference in rate of weight gain is out of proportion to the difference in food intake in the two groups (Fig. 1). The food intake of the group of animals receiving morphine is somewhat less than that of the other group of animals during the first 2 weeks but after this time is equal to, or greater than that of the control animals. The normal animals consistently utilized much more food for growth than did the animals receiving morphine. This is shown by the values obtained for grams of weight

gain per day per gram of food consumed per kilogram of body weight.

Before administration of morphine was begun in the one group of rats, the mean basal metabolic rates in the two groups were approximately the same (Table I) and there was no statistically significant difference between the 2 ( $t$  of dif. = 0.664). At the end of 8 weeks the values in both groups dropped significantly. However, the fall in the mean B.M.R. of the chronically morphinized rats at the 48 hour withdrawal period was only 47.50% as great as that obtained in the control group and was significantly higher ( $t$  of dif. = 3.824) than that of the normal animals at that time. It will also be noted that whereas the mean R.Q. of the normal rats decreased slightly over the 8 weeks period, that of the chronically morphinized rats fell more markedly and significantly ( $t$  of dif. = 3.41).

**Discussion.** There is little information in the literature in regard to the effects of chronic morphine poisoning and withdrawal of the drug on weight change, food utilization and metabolic rate of the rat. Myers and Flynn<sup>7</sup> administered 1 to 5 mg/kg/day to young growing rats over a period of 55 days, with only a slight impairment of growth. If higher doses had been given, it is probable more pronounced effects might have been obtained. Sollmann<sup>8</sup> had previously reported that feeding morphine in the diet in doses increasing from 0.01 to 35 mg/kg/day over a period of 21 weeks increased the rate of growth of young rats during the first 15 weeks. The significance of such results is questionable since there were only two animals in the experimental group. Hildebrandt<sup>9</sup> poisoned rats with morphine, beginning with a dose of 100 mg/kg/day and increasing rapidly to 600 to 1000 mg/kg/day. After two weeks his experimental animals showed a marked weight loss. It is surprising, in view of the fact that he

<sup>7</sup> Myers, H. B., and Flynn, J. B., *Proc. Soc. Exp. Biol. and Med.*, 1928, **25**, 786.

<sup>8</sup> Sollmann, T., *J. Pharm. and Exp. Therap.*, 1924, **23**, 449.

<sup>9</sup> Hildebrandt, F., *Arch. Exp. Path. Pharmacol.*, 1932, **92**, 68.

# 17071. Influence of Chronic Morphine Poisoning on Growth and Metabolism of the Albino Rat.\*

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The appearance of signs of hyperirritability following withdrawal of morphine in certain animal species which have received the drug for a prolonged period of time is fairly well established. Plant and Slaughter<sup>1</sup> and Barbour, Porter and Seelye<sup>2</sup> have presented evidence which might suggest that in the dog these phenomena are related to an increased basal metabolic rate. In line with a conception relating withdrawal signs to an elevated metabolism of tissues is the observation by Shideman and Seever<sup>3</sup> that skeletal muscle obtained from rats chronically poisoned with morphine exhibits an increased oxygen consumption which attains a maximum value 48 hours following withdrawal of the drug. At this time oxygen uptake is approximately twice that of muscle obtained from normal animals. The presence of such a marked increase in the metabolism of skeletal muscle *in vitro* should be reflected in an elevated basal metabolic rate of the animal during the withdrawal period. On the other hand, the presence of certain factors existing *in vivo* (neural and humoral) might mask this increased activity which would become apparent only when relieved of these factors. Swann<sup>4</sup> stated that, during addiction in the rat, morphine has a specific dynamic action on metabolism but that withdrawal is followed by a violent depression of the B.M.R. In order to obtain more information on the

metabolic effects of addiction to morphine and its withdrawal in the rat, the following experiments were performed.

**Experimental.** Two groups of albino rats (12 in each group), averaging approximately 100 g in weight were placed on a diet of ground Purina chow for a period of 9 days. During this period individual food intake, and weight change were determined. The B.M.R. for each animal was obtained by measuring respiratory exchange over two 30 minute periods in a closed system maintained at 29°C in a constant temperature water bath. Air, to which oxygen was added as required, was continually circulated through the system. Oxygen consumption was measured by means of a small spirometer and carbon dioxide was absorbed in alkali which was analyzed for carbonate before and after each experimental period. Surface area for each animal was calculated by the formula of Diack.<sup>5</sup>

Following this control period one group of animals was chronically poisoned with morphine as described previously.<sup>6</sup> When the dose of morphine had reached 200 mg/kg (over a period of 8 weeks) and been maintained at that level for at least a week the drug was discontinued and the B.M.R. again determined on each rat in this group as well as in the control groups. During the fourth week of the experiment, one of the animals receiving morphine died and any previous results obtained on that animal were not used in determining means and in statistical analysis.

**Results.** At the beginning of the experiment the mean weight of each group of animals was approximately the same (Fig.1). During the control period, prior to the administration of morphine, the average gain in

\* This investigation was supported by a Research Grant (M. H. Seever) from the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service.

<sup>1</sup> Plant, O. H., and Slaughter, D. H., *J. Pharm. and Exp. Therap.*, 1936, **58**, 417.

<sup>2</sup> Barbour, H. G., Porter, J. A., and Seelye, J. M., *J. Pharm. and Exp. Therap.*, 1939, **65**, 332.

<sup>3</sup> Shideman, F. E., and Seever, M. H., *J. Pharm. and Exp. Therap.*, 1942, **74**, 88.

<sup>4</sup> Swann, H. G., *Am. J. Physiol.*, 1941, **133**, 467.

<sup>5</sup> Diack, S. L., *J. Nutrition*, 1930-31, **3**, 289.

<sup>6</sup> Shideman, F. E., and Seever, M. H., *J. Pharm. and Exp. Therap.*, 1941, **71**, 383.

cium-poor diet. According to Marriot's report the hypophosphite anion is largely excreted unchanged; for that reason, the hypophosphite P was regarded as non-available in our first tests. When the early observations suggested that this might not be the case, a study of the influence of readily available P and hypophosphite P at varying Ca/P ratios was included.

*Experimental.* Groups of 7 to 8 Sprague-Dawley rats were placed on a diet prepared according to Sobel, Roekenmacher and Kramer,<sup>2</sup> which consists of 70 parts yellow corn meal, 16 wheat gluten, 10 brewer's yeast, and 1 NaCl with the addition of cod liver oil to supply 0.1 unit vitamin D per gram. Analysis showed a content of 0.03% Ca and 0.33% P, which is in accord with the figures given by these authors. All animals were kept for 14 days on this diet as a depletion period, and the control group for an additional 25 days. Additions of calcium hypophosphite, gluconate and secondary phosphate were used in the first comparative tests (A to E in table). Nucleic acid was used in some groups to equalize the content in available phosphorus without adding a phosphate. The additions in groups F to O served to vary the Ca/P ratios as widely as the P content in the basic diet would permit, with proper distinction of P, known to be utilized, and hypophosphite P.

The rats at the beginning of the depletion period were of an age specified for vitamin D assay in the U.S.P. It turned out that the range of permissible variations in weight and age, as set for that test, was too wide than was best suited for these experiments. The rats of the last group were apparently a few days older (Nos. A<sub>3</sub> to O), and consequently had larger calcium reserves. For that reason, each group can be compared only with its own control group. The animals were weighed at the beginning and end of the 25 days' test period, then killed and the femora dissected. The bones, dried at 80°, were freed of all adhering tissue, weighed, incinerated and the ash determined.

*Results.* It is evident that the assimilation

of calcium is about the same whether supplied as hypophosphite, gluconate, phosphate or carbonate. While 130 mg Ca per 100 g diet is inadequate for maximal calcification of the bones, 530 mg gave results equal to 730 and 1030 mg as shown in Groups I to O. The final body weight at the end of the test period was within the same range in all groups disregarding the calcium intake, with exception of group A<sub>1</sub>. While it is possible that the smaller animals were more sensitive to Ca deficiency, it must be pointed out that group B, with equally low Ca feeding, showed only a slight depression in weight. The weight and ash content of the femora reflect more definitely any calcium deficiency than the body weight.

The experiments on Ca/P ratio were limited by the 330 mg P content in the basic diet, which was found to be high enough to prevent any acute P deficiency. As a consequence the results appear to be exclusively a function of the Ca supply. A ratio of Ca/P of 1:2.54 (Group F) was positively not detrimental to bone formation, and neither was one of 1:4.9, (Group H) assuming that hypophosphite P is utilized. This, however, we were unable to prove because of the impossibility to produce a P deficiency with this diet and of the latitude in tolerated Ca/P ratios.

Shohl<sup>3</sup> has claimed that a low Ca/high P ratio lowers the ash content in the bones of the rats. He used, indeed, a ratio of 1:16, but only by sacrificing the absolute Ca content in the diet. When 0.12% Ca was given with 2% P the ash was 36%, but with 0.12% Ca and 0.5% P the ash was the same. This is in keeping with our conclusion that the quantity of Ca and not the Ca/P ratio is the limiting factor. Since, according to Shohl's statement, excessive quantities of phosphorus are toxic, it is difficult to prove a detrimental effect of a low Ca/P ratio at an adequate Ca intake.

Some of the ulnae and radii of the A<sub>1</sub> to E groups were examined by the Ag line test as used in vitamin D assay, but no differences were detected.

It seems that bone formation is not the most

<sup>2</sup> Sobel, A. E., Roekenmacher, M., and Kramer, B., *J. Biol. Chem.*, 1945, **158**, 475.

<sup>3</sup> Shohl, Alfred, *J. Nutr.*, 1936, **11**, 275.

TABLE I.  
Effect of Withdrawal Following Chronic Morphine Poisoning on the Respiratory Quotient and Metabolic Rate of the Rat.

	No. of animals	Initial		Final	
		BMR	R.Q.	BMR	R.Q.
Normal	12	51.5 ( $t = 55.6$ )	.829 ( $t = 53.9$ )	41.95 ( $t = 29.4$ )	.812 ( $t = 36.2$ )
Chronic Morphine	11	53.3 ( $t = 29.2$ )	.830 ( $t = 48.08$ )	48.86* ( $t = 44.1$ )	.764* ( $t = 94.8$ )

\* Values obtained 48 hours after withdrawal of the drug which had been administered in increasing doses (from 20 mg/kg/day to 200 mg/kg/day) over a period of 8 weeks.

was using larger doses and younger rats than were employed in these experiments, that the inhibition of growth was not noted sooner.

It is difficult to interpret the changes in basal metabolic rate of the two groups of animals in the experiments reported here. If the final values were the only ones available to compare, it could be concluded that during the withdrawal period there was an increase in metabolism. However, in view of the fact that the metabolic rate of both groups decreased and since the decrease in each is roughly proportional to the gain in weight, it is possible the final difference is merely due to a difference in weight between the two groups. Nevertheless, the lower respiratory quotient and decreased utilization of food for body growth might indicate an increase in

metabolic rate which is not apparent from the values obtained for basal metabolic rates. Further experiments are planned to clarify the questions provoked by these results.

*Summary.* Chronic administration of morphine in doses increasing from 20 mg/kg/day to 200 mg/kg/day over a period of 8 weeks to young albino rats resulted in a marked suppression of growth. This occurred in spite of a greater food intake per unit of body weight. In addition, the animals receiving morphine demonstrated a significant reduction in their respiratory quotients at the 48 hour withdrawal period. The interpretation of the changes in metabolic rate is not apparent on the basis of the results obtained.

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## 17072. Value of Calcium Hypophosphite and Other Calcium Compounds as Calcium Supplements in Calcium-Low Diets.

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The value of hypophosphites as therapeutic agents has never been objectively demonstrated and Marriott's criticism of their use<sup>1</sup> has largely been accepted. However, calcium hypophosphite is well worth while being considered as a means of convenient calcium administration, where such therapy is desired, because it is high in this element (23%), does not have the objectionable taste of most

calcium salts and, in contrast to gluconate, is adequately soluble. The hypophosphite ion, as a stable acid, balances the alkalinity of the calcium, which effect persists even if there should occur a transformation into phosphoric acid. Therefore, the salt does not shift the acid-base equilibrium to the alkaline side as is the case with salts of carbonate producing acids. The original purpose of the present investigation was a comparison of calcium hypophosphite with dicalcium phosphate and calcium gluconate as a supplement in a cal-

<sup>1</sup> Marriott, McKim, *J. Am. Med. Assn.*, 1916, 70, 486.

sensitive indicator of a deficiency or unbalance of Ca and P. Most of the control rats developed a considerable degree of baldness. This was still more accentuated in Group B, which points to a further damage caused by the change in the Ca/P ratio from 1:11 to 1:13.6. It also appeared markedly in Group K, receiving calcium gluconate without additional P, but was absent in E, where the gluconate was balanced with P. None of the other groups showed loss of hair. By comparing the Ca/P ratio in Group K (1:0.32) with those of the other groups showing baldness (Ca/P, 1:11; 1:13.6) it may be concluded that the disproportion in Ca/P ratio to either extreme has a similar effect. The non-appearance of baldness in Group I may point to some assimilation of hypophosphite P, since otherwise this group would have the

same Ca/P ratio as K. More data will be required to make a positive statement on this point.

*Summary.* Calcium hypophosphite is well suited to serve as a supplement for dietary calcium. To what extent the hypophosphite phosphorus is utilized by the organism remains uncertain. The ratio Ca/P in the diet may be varied within a wide range without affecting the calcification of the bones, provided the absolute quantities of each constituent are adequate. Extreme disproportion of Ca and P in either direction seems to cause loss of hair, as was observed in rats receiving either very low or very high relative levels of calcium.

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### 17073. Extra-Renal Removal of Hemoglobin from Circulation in the Rat. I. Effect of Parenteral Bovine Albumin.

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Hemoglobin in the circulation, whether resulting from hemolysis of erythrocytes *in vivo*, or introduced by parenteral injection, is removed at least in part by excretion through the kidney. Hemoglobin filtered through the glomerulus may appear in the urine, although part is reabsorbed by the proximal tubule cells.<sup>1-3</sup> That hemoglobin is removed by extra-renal routes, as well, may be inferred from

the accumulation of hemosiderin in reticulo-endothelial cells, when erythrocytes are destroyed at an abnormally rapid rate.

Parenteral injections of albumin might be anticipated to alter the rate at which hemoglobin is removed from the circulation by extra-renal routes. Since injected albumin is itself removed rapidly from the circulation, a nonspecific increase in protein removal might occur. On the other hand, the mechanism for protein homeostasis might be so saturated that the rate of hemoglobin removal might diminish. Overloading the reabsorption mechanism with bovine albumin is known<sup>4</sup> to affect the renal tubule cells so as to diminish reabsorption of hemoglobin from tubular urine.

Our interest in the extra-renal removal of hemoglobin from the circulation arose during an investigation into the mechanism of proteinuria. Using hemoglobin as an indicator,

\* The author gratefully acknowledges the assistance of Helen J. Ureen. This work was supported by a grant from the Division of Research Grants and Fellowships, National Institutes of Health, U. S. Public Health Service. Bovine albumin (Fraction V) was supplied through the courtesy of Dr. J. D. Porsche, Armour Laboratories, Chicago, Ill. Hemoglobin was furnished through the courtesy of Dr. R. B. Pennell, Sharp and Dohme, Inc., Glenolden, Pa.

<sup>1</sup> Yuile, C. L., *Physiol. Rev.*, 1942, 22, 19.

<sup>2</sup> Gerard, P., *J. Anat. and Physiol.*, 1936, 70, 354.

<sup>3</sup> Rather, L. J., *J. Exp. Med.*, 1948, 87, 163.

<sup>4</sup> Lippman, R. W., *Am. J. Physiol.*, 1948, 154, 532.

TABLE I.

	Addition to basic diet	Mg Ca per 100 g	Mg P per 100 g	Ratio, Ca/P	Wt. at beginning of test period	end	Femora wt., mg	Ratio femora mg to wt. g	% ash in femora
A <sub>1</sub>		30	330	1:11	68.0 ±7.6	91.0 ±10.8	301.5 ±22.5	3.13 ±0.51	31.24 ±1.71
B	Nucleic acid	30	330	1:13.6	72.5 ±10.1	110.1 ±14.5	326.5 ±16.4	2.91 ±0.30	31.45 ±0.08
C	CaHPO <sub>4</sub> 2H <sub>2</sub> O	30 100	330 77	1:3.13	60.7 ±3.2	132.8 ±8.8	352.7 ±14.6	2.97 ±0.13	41.73 ±1.74
D	Ca(H <sub>2</sub> PO <sub>4</sub> ) <sub>2</sub> Nucleic acid	30 100	330 77	1:3.13	64.0 ±3.0	134.9 ±4.4	395.6 ±18.8	2.93 ±0.15	40.76 ±1.08
E	Ca gluconate Nucleic acid	30 100	(155) 330	(1:4.3) 1:3.13	61.1 ±3.8	115.6 ±9.0	378.7 ±23.0	3.38 ±0.18	41.86 ±1.53
A <sub>2</sub>		30	330	1:11	83.9 ±7.8	131.7 ±7.9	268.4 ±16.5	2.04 ±0.08	30.70 ±0.40
F	Ca(H <sub>2</sub> PO <sub>4</sub> ) <sub>2</sub>	30 100	330 (155)	1:2.54 (1:3.73)	83.2 ±9.0	135.1 ±13.8	341.5 ±27.5	2.54 ±0.17	39.22 ±1.27
G	Ca gluconate	30 100	330	1:2.54	88.4 ±10.8	135.9 ±9.8	329.3 ±32.9	2.42 ±0.19	39.06 ±1.01
H	Ca(H <sub>2</sub> PO <sub>4</sub> ) <sub>2</sub> Na(H <sub>2</sub> P <sub>2</sub> O <sub>7</sub> ) H <sub>2</sub> O	30 100	330 (155)	1:2.54 (1:4.9)	83.5 ±3.1	131.4 ±9.2	324.6 ±17.2	2.48 ±0.12	40.49 ±1.29
A <sub>3</sub>		30	330	1:11	95.2 ±7.7	129.3 ±10.7	231.2 ±39.7	2.56 ±0.23	36.9 ±1.2
I	Ca(H <sub>2</sub> PO <sub>4</sub> ) <sub>2</sub>	30 1000	330 (1550)	1:0.32 (1:1.8)	98.0 ±8.4	121.2 ±10.7	472.6 ±45.1	3.93 ±0.12	52.5 ±1.2
K	Ca gluconate	30 1000	330	1:0.32	97.2 ±5.0	138.8 ±19.1	466.9 ±59.4	3.65 ±0.29	51.6 ±2.0
L	Nucleic acid Ca(H <sub>2</sub> PO <sub>4</sub> ) <sub>2</sub>	30 1000	330 (1550)	1:0.61 (1:2.0)	96.7 ±6.4	125.2 ±9.3	476.6 ±24.6	3.82 ±0.24	53.0 ±0.8
M	Nucleic acid Ca(H <sub>2</sub> PO <sub>4</sub> ) <sub>2</sub>	30 700	330 300	1:0.9	95.6 ±2.8	125.3 ±11.3	458.9 ±35.7	3.66 ±0.18	52.4 ±1.1
N	Ca(H <sub>2</sub> PO <sub>4</sub> ) <sub>2</sub>	30 330	(1085)	(1:2.4)	101.4 ±7.6	140.3 ±13.6	504.2 ±44.9	3.60 ±0.24	53.2 ±1.6
O	CaCO <sub>3</sub>	30 1000	(775) 330	(1:2.09) 1:0.32	90.8 ±6.4	132.8 ±8.2	462.1 ±61.2	3.48 ±0.10	53.4 ±2.2

In the columns of Ca and P values, the first figure represents the quantities contained in the basic diet, the following figures are added Ca and P. The parentheses signify hypophosphite P, or in the Ca/P ratio, the inclusion of hypophosphite P in the total P value. The second figures in the data of results are standard deviations  $S = \pm \sqrt{\frac{\sum(d^2)}{n-1}}$ .

TABLE I.  
Rate of Removal of Hemoglobin from the Circulation of Nephrectomized Rats.

Min. after inj.	Final serum conc. of Hb, mg/100 cc	Wintr. hematocrit	Plasma vol., cc	Total circ. Hb, mg	Hb removed mg	Rate of Hb removal mg/min./100 g BW	Serum Prot.-Hb conc., %	Total circ. Prot.-Hb, mg
Sodium Chloride injections								
2	1492	35.3	9.5	141	0		3.54	336
30	1398	37.9	8.9	124	17	$0.41 \pm .19$	4.53	400
60	1078	38.5	8.8	95	46	$0.50 \pm .06$		
Bovine albumin injections								
2	1403	33.2	10.0	141	0		6.40	640
30	1179	33.5	10.1	119	22	$0.49 \pm .16$	6.56	650
60	1135	37.8	8.8	100	41	$0.44 \pm .08$	7.54	664

36 hours. Control rats in this series had a total circulating protein value (other than hemoglobin) of approximately 350 mg. As the result of injections which introduced approximately 2500 mg of albumin into the circulation during a period of 30 hours (about half of the last injection remained unabsorbed at the time of autopsy), the total circulating protein rose to about 650 mg, nearly twice its previous level. Consequently a minimum of 2200 mg entered and left the circulation during the period of observation. In spite of this, no appreciable change in the rate of hemoglobin removal was detected.

Comparison of the extra-renal rate of removal with the renal excretion of hemoglobin at comparable serum concentrations indicated that, in the rats injected with sodium chloride solution, the renal excretion rate<sup>4</sup> was less than twice the extra-renal removal rate, while in

the rats injected with bovine albumin the renal excretion rate was relatively somewhat greater. The difference in ratio depended upon the effect of bovine albumin injections, increasing the rate of renal excretion.

*Summary.* 1. During the hour following intravenous injection of hemoglobin in the rat, about 1/3 of the quantity injected left the circulation by extra-renal routes.

2. Intraperitoneal injection of bovine albumin, with the associated rapid passage of albumin from the circulation, did not affect the rate at which hemoglobin left the circulation.

3. The rate at which hemoglobin leaves the circulation by extra-renal routes is of comparable magnitude to, though less than, the rate of renal excretion.

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## 17074. Experimental Toxoplasmosis. II. Effect of Sulfadiazine and Antiserum on Congenital Toxoplasmosis in Mice.

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Although toxoplasmosis has been known as an infectious disease of man for over a decade, therapy of the acute condition has remained unsatisfactory. Sabin and Warren,<sup>1,2</sup> testing

<sup>1</sup> Sabin, A. B., and Warren, J., *Proc. Soc. Exp. Biol. and Med.*, 1942, 51, 15.

<sup>2</sup> Sabin, A. B., and Warren, J., *Proc. Soc. Exp. Biol. and Med.*, 1942, 51, 19.

a wide variety of antimicrobial agents, reported that only the sulfonamides have any beneficial effects on the course of the disease in laboratory infected mice and rabbits. Weinman and Berne<sup>3</sup> extended these observa-

<sup>3</sup> Weinman, D., and Berne, R., *J.A.M.A.*, 1944, 124, 6.



we wanted to know the speed with which it left the circulation by extra-renal routes, so that the rate of urinary excretion could be better understood.

The rate of urinary hemoglobin excretion is the resultant of two opposed rates: filtration at the glomerular membrane, and reabsorption by tubular cells. Neither of these may be measured directly. However, the rate at which hemoglobin is removed from the circulation may be measured by determining total circulating hemoglobin (the product of plasma hemoglobin concentration and plasma volume) at suitable intervals. If the kidneys are surgically removed, then hemoglobin can leave the circulation only by extra-renal routes, so that the total rate of hemoglobin removal, which can be measured, becomes equal to the extra-renal rate.

**Methods.** In this study 48 male rats were used, each weighing about 150 g, with little variation. One group of rats received two 16 cc intraperitoneal injections of 6% bovine albumin in 0.85% sodium chloride solution at 9:00 A.M. and 4:30 P.M. on the day preceding the experiment. At 9:00 on the day of the experiment they received another such injection, and 5½ hours later they were subjected to bilateral nephrectomy under light ether anesthesia. The operative procedure was performed rapidly through 2 flank incisions. The kidney was delivered through the incision, a silk ligature was placed around the renal pedicle, and the pedicle severed. The wound was closed with Michel clips. Very little blood was lost. Previous experience has shown that rats endure this procedure with no immediate evidence of ill effects. Within a few minutes the rats are awake again, and show normal behavior.

Immediately after the completion of nephrectomy, and while still anesthetized, the rats received an intravenous injection of 141.0 mg of hemoglobin,<sup>5</sup> in a volume of 1.7 cc. Groups of rats were killed at intervals after injection by exsanguination from the abdominal aorta. By previous experience we know

that no hemolysis occurs under the conditions of collection. Determinations were made of the serum hemoglobin concentration, total serum protein concentration, and Wintrobe hematocrit value. The serum protein concentration other than hemoglobin was calculated by difference. Another group of rats was treated in exactly the same manner, but, in place of the injections of bovine albumin, received intraperitoneal injections of 0.85% sodium chloride solution in equivalent volume, without the added protein.

Total hemoglobin concentration was measured by the method of Evelyn and Malloy.<sup>6</sup> Plasma volumes were calculated from the serum hemoglobin concentration and the known amount of hemoglobin injected, in the groups killed at 2 minutes. The small error introduced by the short mixing time has been neglected, as it is of smaller magnitude than other causes of error in such measurements.<sup>7</sup> If we assume that the circulating erythrocyte mass remains constant over short periods of time, the hematocrit value may be taken as an index of plasma volume changes at the 30 and 60 minute intervals, at which times plasma volumes were calculated from the mean initial plasma volume.

Total protein determinations were made by a modification of the Kingsley biuret method.<sup>8</sup>

**Results.** Hemoglobin was removed from the circulation with relatively great rapidity. In 60 minutes, approximately 1/3 of the quantity injected intravenously had disappeared from the circulation (Table I). The injections of bovine albumin did not appear to affect significantly the rate at which hemoglobin left the circulation.

**Discussion.** The speed at which physiological functions involving proteins occur is not generally appreciated. Thus, only recently, Stoerk, John, and Eisen<sup>9</sup> reported that the half-life of serum protein in the rat is about

<sup>6</sup> Evelyn, K. A., and Malloy, H. T., *J. Biol. Chem.*, 1938, **126**, 655.

<sup>7</sup> Lippman, R. W., *Proc. Soc. Exp. Biol. and Med.*, 1947, **66**, 188.

<sup>8</sup> Kingsley, G. R., *J. Biol. Chem.*, 1939, **131**, 197.

<sup>9</sup> Stoerk, H. C., John, H. M., and Eisen, H. N., *Proc. Soc. Exp. Biol. and Med.*, 1947, **66**, 25.

<sup>5</sup> Pennell, R. B., Smith, W. E., and Werkheiser, W. C., *Proc. Soc. Exp. Biol. and Med.*, 1947, **65**, 295.

TABLE I.  
Treatment of Offspring from Non-Immune Mothers.

Group	Treatment begun 3rd day after birth	No. of newborn mice in group	No. of mice dead of toxo- plasmosis	Avg survival period of mice dead of toxo- plasmosis, days	Mice surviving experimental period (1 mo.) %	Survivors found chronic carriers, %
A	Sodium sulfadiazine 0.5 mg/g daily, 8 days	40	22	$6 \pm 3$	45	67
B	Antiserum 0.02 cc daily, 8 days	40	28	$6 \pm 3$	30	83
C	Sodium sulfadiazine 0.5 mg/g daily plus antiserum 0.02 cc daily, 8 days	40	5	$6 \pm 2$	88	46
D Controls	None	40	35	$4 \pm 3$	13	60

Group A: 40 newborn mice treated 8 days with sodium sulfadiazine, 0.5 mg/g of body weight.

Group B: 40 newborn mice treated 8 days with 0.02 cc antiserum per day.

Group C: 40 newborn mice treated with 0.5 mg/g sodium sulfadiazine per day plus 0.02 cc antiserum per day for 8 days.

Group D: 40 newborn mice receiving no treatment.

All treatment was started on the third day after birth. This day was chosen since widespread dissemination of *Toxoplasma* through the tissues was almost invariably present by this time.

The antiserum was obtained from mice hyperimmunized by the injection of gradually increasing doses of *Toxoplasma* F strain\* at bi-weekly intervals, followed by increasing doses of the more virulent RH strain at weekly intervals until the mice were resistant to at least 100 minimum lethal doses of the RH strain. Two weeks after the last injection, the animals were bled, and the serum frozen at  $-22^{\circ}\text{C}$ . Each day the required quantity was thawed and promptly injected intraperitoneally. Enough serum was prepared so that the en-

tire experiment could be performed with one batch. Grace had shown previously that immune serum from rabbits also has a beneficial effect on the course of the infection in adult mice;<sup>9</sup> we preferred to employ mouse serum in order to avoid introducing the additional complicating factor of foreign protein reactions.

Sulfadiazine was injected subcutaneously as a 0.5% solution of the sodium salt dissolved in physiological saline solution. The daily dosage of 0.5 mg per gram of body weight of mouse was divided into 3 doses given at approximately 8 hour intervals. This dosage gives blood levels similar to those achievable safely in man, averaging about 15 mg/100 cc blood one hour after injection, 12 mg after 3 hours and 7 mg after 6 hours, as measured by the methods described by Bratton and Marshall.<sup>10</sup> These levels produced no apparent toxic effects in mice. The results are shown in Table I.

One month after birth, all survivors were examined for the presence of *Toxoplasma* by microscopic observation of Giemsa-stained material and by subinoculation of tissue suspensions into fresh mice.

Pathologically, the picture presented by the mice treated with sulfadiazine alone dif-

\* Obtained through the kindness of Dr. Isabella B. Grace of the Department of Public Health and Preventive Medicine, Cornell University Medical College.

<sup>9</sup> Grace, I. B., personal communication, 1947.

<sup>10</sup> Bratton, A. C., and Marshall, E., *J. Biol. Chem.*, 1939, **128**, 537.

tions, and by producing higher blood levels of the drugs with exceedingly large doses of sulfonamides were able to show that if used early in the course of the infection these agents are effective in curing the acute phase of the disease in adult mice. However, this method of therapy did not eradicate the infection; all treated mice remained chronic carriers, retaining virulent organisms in the brain.

In man, sulfonamide therapy for toxoplasmosis has so far given striking results in only one of the few cases in which it has been employed.<sup>4-6</sup>

In practice, therapy of the infection is handicapped by the fact that most recognized cases occur in newborn infants who have acquired the infection *in utero*. In consequence, widespread, irreparable, and frequently fatal damage to tissues has occurred even before birth.<sup>7</sup> However, we have recently observed a number of infants who seemingly had acquired the infection shortly before term, so that at the time of delivery there appeared to be little demonstrable tissue destruction. It is for these children that a successful method of treatment would be of particular value.

**Experimental Procedures.** The *Toxoplasma* strain used was the same RH strain described in an earlier paper.<sup>8</sup> Although it is possible to produce congenital toxoplasmosis in mice similar to that described in man, under the experimental conditions employed only about 30% of the offspring from an infected mother could be shown to harbor *Toxoplasma*. Of these, about three-fourths died; in the remaining infected animals the disease went on to a chronic carrier state. Because of the relatively small percentage of congenital infections produced by this method, very large numbers of animals would have been required to evaluate adequately the effect of any therapeutic agent. Therefore, in the present investigation an artificial, rather than

natural, method of infecting the embryo was employed.

Adult, virgin, female mice were mated with healthy males and as soon as a vaginal plug appeared, a sign of successful copulation, were removed to individual cages. Fourteen days after copulation they were examined for signs of pregnancy. The pregnant animals were anesthetized with ether, the lower abdomen shaved, and a small incision made through the skin and peritoneum to expose the uterus. Aseptic technic was used throughout. The position of the various embryos in the uterus could easily be seen. With some practice, it was possible to insert a very fine hypodermic needle through the walls of the uterus and the fetal membranes so that it just touched the embryo. The infective material was then injected through a 0.5 cc tuberculin syringe attached to the needle. This material consisted of 0.02 cc of a 1:100 dilution of peritoneal exudate harvested from an infected, moribund, adult mouse. Each embryo was individually injected. The peritoneum was then closed with interrupted fine silk sutures, the skin with continuous silk sutures, and the wound covered with a collodion dressing.

As will be seen below, it was possible by this method to produce an infection active at birth in over 90% of the embryos injected. The pathological picture in these animals was indistinguishable from that described for those infected by transplacental transmission, except that in the latter group the degree of parasitization appeared less and the fatality rate was lower.

One hundred sixty embryos were injected on the 14th day in the manner outlined above, and the mothers allowed to give birth, which in almost every case occurred on the 18th and 19th day of gestation. Within 10 hours after birth, the offspring were separated from their mothers, divided arbitrarily into litters of 5, and placed in cages with healthy lactating foster mothers. This was done in order to eliminate the possibility of milk-borne transmission of *Toxoplasma* during the period of therapy since about 50% of the mothers of the injected embryos developed toxoplasmosis.<sup>8</sup> The litters were then divided into the following 4 groups:

<sup>4</sup> Sabin, A. B., *J.A.M.A.*, 1941, **116**, 801.

<sup>5</sup> Zuelzer, W. W., *Arch. Path.*, 1944, **38**, 1.

<sup>6</sup> Robinson, P., *Ann. Paediat.*, 1947, **138**, 134.

<sup>7</sup> Cowen, D., Wolf, A., and Paige, B. H., *Arch. Neurol. and Psych.*, 1942, **48**, 689.

<sup>8</sup> Eichenwald, H., *Am. J. Dis. Child.*, 1948, **70**, 307.

immune mothers. This group was given no treatment. The therapy given to the test groups was identical to that used in the previous experiment. The results are shown in Table II.

The data in Table II indicate that antibodies passively acquired transplacentally by the fetus are not sufficient to protect the offspring even when sulfadiazine is given after birth, and that additional antiserum must be supplied to the progeny for optimal therapeutic effects.

Two other drugs<sup>†</sup> were also tried alone and in combination with antiserum but failed to show any beneficial action. These were "Promizole" (4,2' diaminophenyl 5' thiazole sulfone) given to newborn mice in a dosage of 0.5 mg/g per day for 8 days and "Diazone" (disodium formaldehyde sulfoxylate diaminophenyl sulfone) given in 0.1 mg/g per day for 8 days.

*Discussion.* A comparison of the results obtained by the various forms of treatment, as listed in Table I, readily shows that while sulfadiazine and antiserum each have a beneficial action on the course of toxoplasmosis, a combination of these two substances is considerably more effective than the use of either material alone. The reasons for this fact are probably many: one, already mentioned, may be the difficulty encountered in trying to obtain high antibody concentrations in the central nervous system; another may be the fact that humoral antibodies do not appear to have any action on intracellular *Toxoplasma*,<sup>13</sup> while extracellular organisms are not affected by sulfonamides.<sup>1</sup>

<sup>†</sup> Obtained through the kindness of Dr. Walsh McDermott of the Department of Medicine, The New York Hospital and Cornell Medical College.

It should be pointed out that the histological structure of the placenta in man and in the mouse is similar if one considers the fact that in both cases only a single layer of cell separates the maternal from the fetal blood.<sup>14</sup> The thickness of this layer is believed to be of importance in transplacental antibody transfer.<sup>15</sup>

We have observed several cases of human congenital toxoplasmosis in whom, when tested a few days after delivery, the mother showed a higher neutralizing titer to *Toxoplasma* than did the infant. This suggests that perhaps the transfer of antibody from mother to fetus through the placental barrier is not complete, which may be one reason why the embryos from highly immune female mice are susceptible to the infection.

Even though one must be cautious not to apply unselectively data obtained from animal experimentation to human diseases, it would appear that the combined antiserum and sulfadiazine therapy should be given a clinical trial in active human congenital cases.

*Summary.* The effect of sulfadiazine, antiserum and a combination of these two materials on the course of congenital toxoplasmosis in mice was determined. It was shown that while sulfadiazine and antiserum individually had some beneficial action, a combination of these substances was considerably more effective than either used alone.

<sup>13</sup> Sabin, A. B., and Feldman, H. A., *Science*, 1948, **108**, 660.

<sup>14</sup> Mason, J. H., Dalling, T., and Gordon, W. J., *Path. Bact.*, 1930, **33**, 783.

<sup>15</sup> Schneider, L., and Szathmary, J., *Z. Immunforsch.*, 1940, **98**, 24.

TABLE II.  
Course and Treatment of Offspring from Immune Mothers.

Group	Treatment begun 3rd day after birth	No. of newborn mice in group	No. of mice dead of toxo- plasmosis	Avg survival period of mice dead of toxo- plasmosis, days	Mice surviving experimental period (1 mo.) %	Survivors found chronic carriers, %
A	Sodium sulfadiazine 0.5 mg/g daily, 8 days	40	13	7 ± 3	68	70
B	Antiserum 0.02 cc daily, 8 days	40	22	7 ± 3	45	78
C	Sodium sulfadiazine 0.5 mg/g daily plus antiserum 0.02 cc daily, 8 days.	40	4	6 ± 1	90	53
D Controls from im- mune mothers	None	40	26	6 ± 3	35	82
E Controls from non- immune mothers	None	40	33	4 ± 2	18	71

ferred considerably from that observed in the animals treated with antiserum alone. In the latter case, active inflammatory lesions were most marked in the brain, and only relatively few scattered, apparently inactive lesions could be found in the other viscera. In the animals given sulfadiazine there were many scattered lesions throughout the liver, lung, spleen, brain, and kidney. These, however, were considerably smaller in both size and number and showed markedly less necrosis and inflammatory reaction than the widespread granulomata noted in the untreated control series. The persistence of active lesions in the central nervous system after treatment with antiserum may, perhaps, be explained best by the slight permeability of cerebral capillary walls to immune bodies, as postulated by Friedeman.<sup>11</sup> It was not feasible to use larger doses of antiserum.

As indicated on Table I, in the mice treated with combined antiserum and sulfadiazine, approximately half of the survivors had been

cured. The remainder continued to harbor parasites, mostly in the brain, and had thus become chronic carriers.<sup>12</sup> The mice that died during treatment showed a widespread parasitization of tissues; in these cases the infection apparently had already been overwhelming before therapy was started.

In order to evaluate the effects of maternal antibodies on the course of toxoplasmosis in the embryo and in the newborn mouse, adult virgin female mice were injected subcutaneously with a sub-lethal dose of *Toxoplasma* F strain. This produced a transient disease which was followed in about 3 weeks by a strong immunity to a challenge dose of RH strain. Two weeks later the immune animals were allowed to mate with healthy male mice. On the 14th day of pregnancy, the embryos were injected *in utero* as described above. After birth, the litters were again given healthy foster mothers, and divided into 4 groups as before, plus an additional group E consisting of 40 newborn mice born from non-

<sup>11</sup> Friedeman, U., *Physiol. Rev.*, 1942, **22**, 125.

<sup>12</sup> Weinman, D., *J. Infect. Dis.*, 1943, **73**, 85.

TABLE I.  
Effect of Concentration of Saline on Infectivity of Suspension for Embryos and Red Cell Agglutinin.

Exp.	Conc. of saline	.8			2%			3%			4%			Untreated
		Embryo infect.	Red cell titer	Embryo infect.	Embryo infect.	Red cell titer	Embryo infect.	Embryo infect.	Red cell titer	Embryo infect.	Embryo infect.	Red cell titer	Embryo infect.	
1	Immediate	9.0		8.0				9.4			8.8			8.5
	9 days at 4°C	7.5									8.8*			
											7.6			
2	Immediate		1280			1280			640			640		
	7 days at 4°C	7.8		8.3		2560			1280		7.4	640		

\* Duplicate titration.  
All titrations were carried out as in previous studies.<sup>6</sup>

though there is no detectable loss of activity (red cell agglutinating or embryo infectivity) of the virus.

If a 2% osmic acid solution was added to the .15M saline suspension of the virus no change in shape was detected (Fig. 1). It remained spherical. If the same .15M saline suspension of virus was dried slowly in the cold room at 4°C instead of at room temperature, again filamentous forms were not detected. This is in contrast to drying at room temperature (Fig. 2). This made it likely that a change in shape was taking place during the drying process. When we combined this idea with the assumption that osmic acid fixed the virus in the form in which it existed in solution we were led to the following experiments.

Virus freshly harvested from allantoic fluid was centrifuged at about 15,000 g for 40 minutes in an angle centrifuge. It was then resuspended in varying concentrations of saline (Table I). It was washed again by a repeated centrifugation and resuspension in the same concentration of saline. All of the preparations were then "fixed" by exposure to the osmic acid in solution. They were then transferred back to water by a third centrifugation. All of the preparations for electron microscopy were then similarly prepared by drying a drop of this final water suspension on the usual formvar screen, washing, and shadowing with chromium at angle of 4 to 1.

Figs. 3 and 4 show the change in shape which has then been detected in four separate experiments with 2 strains of the virus. We have not as yet determined the concentration of saline at which the change in shape will occur or the quantitative effect of different concentrations on different morphologies, but have shown that a marked change in shape occurs at 2% saline which is 2.5 the normal concentration of saline. This despite the lack of any evidence that the embryo infectivity was detectably reduced by placing the virus in even four per cent saline. Furthermore, the rate of loss of infectivity of the virus on remaining in the ice box was the same in different concentrations of saline.

<sup>6</sup> Bang, F. B., *J. Exp. Med.*, 1948, **88**, 233.

# 17075 P. Formation of Filamentous Forms of Newcastle Disease Virus in Hypertonic Concentration of Sodium Chloride.\*

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The shape of the virus of Newcastle disease of chickens is filamentous when prepared from a saline suspension<sup>1-3</sup> and roughly spherical when prepared from allantoic fluid or water.<sup>4</sup> If the virus is partially inactivated while in water, it does not change shape when the saline is brought to .15M. This concentration of salt is sufficient to cause a change in the

form of the active virus when such a suspension is dried on an electron microscope screen.<sup>5</sup>

Two recent chance observations added further qualifications to the system, and with subsequent experiments further indicate that the change in shape takes place in solution. A hypertonic concentration of saline is, however, needed to cause the change in shape, al-

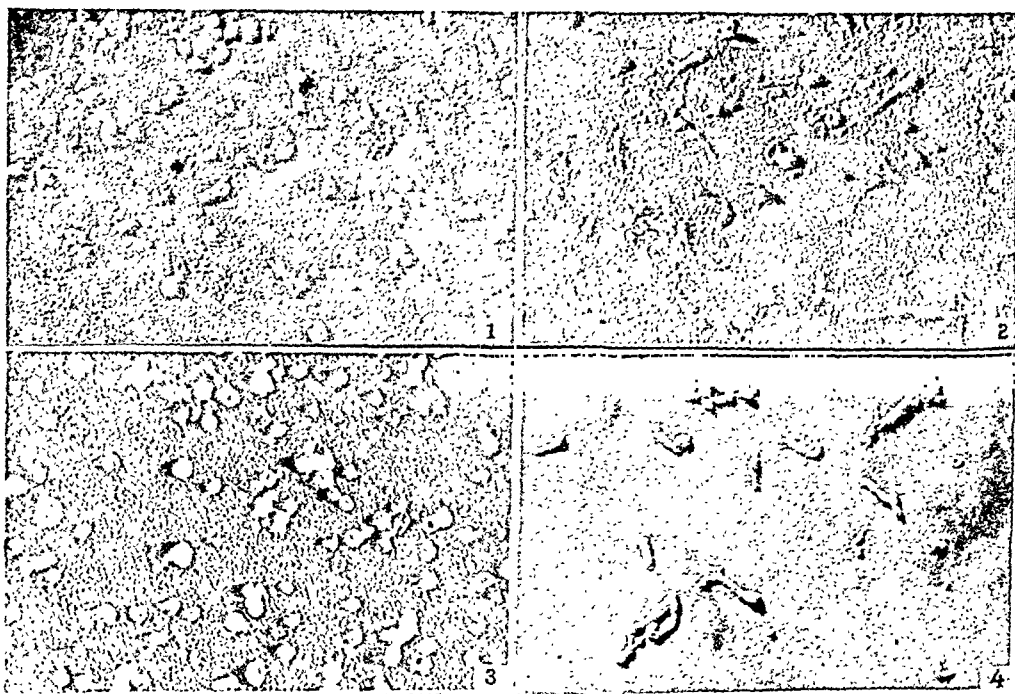


FIG. 1. Fixed in .15 M. saline by exposure to osmic acid before drying on electron microscope screen.

FIG. 2. Dried from similar suspension without fixation.

FIG. 3. Exposed to 3% saline by 2 centrifugation-washing cycles. Put back in water before preparation of screen.

FIG. 4. Same as 3, except fixed in osmic acid while in 3% saline.

\* This study was supported in part by a grant-in-aid from the National Institutes of Health, U. S. Public Health Service.

1 Bang, F. B., *PROC. SOC. EXP. BIOL. AND MED.*, 1946, **64**, 135.

2 Cunha, R., Weil, M. L., Beard, D., Taylor, A. R., Sharp, D. G., and Beard, T. W., *J. Im-*

*munol.*, 1947, **55**, 69.

3 Elford, W. J., Smiles, J., Chu, C. M., and Dugdon, J. A., *Biochem. J.*, 1947, **41**, XXV.

4 Bang, F. B., *PROC. SOC. EXP. BIOL. AND MED.*, 1947, **64**, 135.

5 Bang, F. B., *J. Exp. Med.*, 1948, **88**, 251.

TABLE I.

Integrated Mean Pressures in Pulmonary Artery and Vein in Relation to Heart Rate Changes.

	Wt. of dog, kg		Before vagal stimulation		During first min. of vagal stimulation		After vagal stimulation	
			beats/min. mm/Hg		beats/min. mm/Hg		beats/min. mm/Hg	
1	15.7	Heart rate	216		72		240	
		Pulmonary venous pressure		4.0		14.8		4.5
		Heart rate	180		60		200	
		Pulmonary artery pressure		12.8		9.3		12.8
2	18.6	Heart rate	144		72		144	
		Pulmonary venous pressure		5.0		9.5		5.3
		Heart rate	144		48		130	
		Pulmonary artery pressure		16.0		14.0		17.3
3	24.2	Heart rate	180		72		180	
		Pulmonary venous pressure		6.3		11.3		6.3
		Heart rate	180		72		180	
		Pulmonary artery pressure		20.0		18.0		19.8
4	20.0	Heart rate	168		60		168	
		Pulmonary venous pressure		9.0		13.8		5.0
		Heart rate	168		60		180	
		Pulmonary artery pressure		16.3		15.3		17.5
Average		Heart rate	178		69		183	
		Pulmonary venous pressure		6.1		12.4		5.3
		Heart rate	168		60		173	
		Pulmonary artery pressure		16.3		14.2		16.9

with sodium pentobarbital, 30 mg/kg and No. 10 radio-opaque whistle-tipped ureteral catheters were put into the jugular vein and carotid artery. The catheters were passed into the pulmonary artery and pulmonary vein respectively with fluoroscopic aid. A small cannula was placed in the thoracic cavity and intrathoracic, pulmonary artery and pulmonary vein pressures were recorded with standard resistance wire pressure transmitters (Statham strain gauges).<sup>†</sup> A more detailed description of the technic employed was reported elsewhere.<sup>4</sup>

Femoral arterial pressure was measured with a mercury manometer, and jugular venous pressure was measured with a manometer filled with 5% sodium citrate. Heart rates were counted from the pulmonary artery pressure records. Integrated mean pulmonary vascular and intrathoracic pressures were measured with a compensating polar planimeter, and all pulmonary pressures reported herein are expressed in relation to intrathoracic

pressure as zero. All dogs had an indwelling tracheal cannula.

A Harvard inductorium was used for faradic stimulation of the cardiac ends of the cut vagus nerves and acetylcholine and/or acetyl-beta-methylcholine were also employed in order to produce bradycardia.

*Results and discussion.* Faradic stimulation of the cardiac ends of the cut cervical vagi was employed to produce cardiac slowing to one-half or one-third of the control rate. There was a prompt elevation of mean pulmonary venous pressure, and a smaller fall in mean pulmonary artery pressure. Details of these observations are presented in Table I. The average values for the 4 dogs studied show a 6.3 mm Hg rise in integrated mean pulmonary venous pressure concomitant with the slowing of the heart rate from 178/minute to 69/minute. Simultaneously with the acute bradycardia in the same dogs the pulmonary artery pressure was lowered 2.1 mm Hg. It may be noted that in Dog No. 1 the integrated mean pressure recorded was higher in the pulmonary vein than in the pulmonary artery. Several factors com-

<sup>†</sup> Statham Laboratories, 9328 Santa Monica Blvd., Beverly Hills, Calif.

<sup>4</sup> Haddy, F. J., Campbell, G. S., Adams, W. L., and Visser, M. B., *Am. J. Physiol.*, in press.



*Discussion.* Since the original description of filamentous and sperm shaped virus particles<sup>1-3</sup> as contrasted with the spherical ones present in allantoic fluid and water we have been puzzled about the shape of the virus under physiological conditions. This is of practical interest in any attempt to study the virus in cells. Two things now seem to be fairly clear. First, the virus is probably spherical when present in solutions of physiological concentrations. This fully explains our previous failure to detect a change in shape by direct measurements of viscosity, light scattering and double refraction of flow.<sup>5</sup> However, it also seems clear that a change in shape will occur in hypertonic salt solutions, for elimination of the salt from the solution returns the virus to its previous shape if it has not been fixed by osmic acid.<sup>†</sup> To the following extent then the filamentous and

sperm shaped virus particles are artifacts. They are formed by the increasing hypertonicity of the salt solution when it dries on the formvar screen. They are, however, real in that they probably exist in free solutions of somewhat hypertonic concentrations. They are at least roughly as active as the spherical forms, and do not lose activity any more rapidly than the spherical forms in water or physiological saline.

*Summary.* In 4 separate experiments it has been shown that the virus of Newcastle disease of chickens probably has a spherical shape when in water or .8% saline. If the concentration of saline is increased to two per cent or more the virus becomes filamentous. It may be fixed with osmic acid at this concentration and will then retain its shape when placed in water. It reverts to the spherical form when replaced in water without previous fixation. No loss of infectivity was detected during these changes in concentration of saline.

† Recent electron microscope observations by Elford *et al.*, *Brit. J. Exp. Path.*, 1948, **29**, 590, also indicate the presence of an osmotic membrane around the virus of Newcastle disease. We are in essential agreement with their results.

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## 17076. Effect of Acute Bradycardia on Pulmonary Vascular Pressures in Anesthetized Dogs.\*

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Pulmonary edema, congestion and hemorrhage were reported in guinea pigs subjected to increased intracranial pressure; however, bilateral cervical vagotomy prior to elevation of intracranial tension resulted in a significantly lesser degree of pulmonary pathology.<sup>1</sup> Luisada and Sarnoff<sup>2</sup> employed massive, rapid venous infusion in dogs simultaneously with vagal stimulation and concluded "electrical stimulation of either the cardiac end of the

cut vagi or the intact nerves favors pulmonary edema by causing extreme bradycardia".

The present authors<sup>3</sup> have reported bradycardia, decreased cardiac output, a significant rise in pulmonary venous pressure and pulmonary edema in dogs subjected to increased intracranial pressure. Intravenous administration of atropine after induction of the changes listed above as following maintained elevation of the intracranial pressure, reversed those changes, and significant pulmonary edema did not occur.

*Methods.* Mongrel dogs were anesthetized

\* This work was supported by a grant from the Life Insurance Medical Research Fund.

1 Campbell, G. S., and Visscher, M. B., *Am. J. Physiol.*, in press.

2 Luisada, A. A., and Sarnoff, S. J., *Am. Heart J.*, 1946, **31**, 282.

3 Campbell, G. S., Haddy, F. J., Adams, W. L., and Visscher, M. B., *Am. J. Physiol.*, in press.

sulfasuxidine was added, failed to grow normally and developed a normocytic anemia. Following treatment with a highly purified antipernicious anemia liver extract, growth was resumed and, after a definite although not marked reticulocytosis, the blood returned to normal. This observation has been confirmed by Cunha *et al.*<sup>2</sup> However, Welch *et al.*<sup>3</sup> fed a similar diet to two 63 day old pigs and failed to observe either retardation of growth or anemia. Cunha *et al.* reported that the development of the anemia was prevented by the administration of pteroylglutamic acid and, to a lesser extent, by antipernicious anemia liver extract.

More recently it has been demonstrated in this laboratory<sup>4,5</sup> as well as by Welch and Heinle,<sup>6-8</sup> that swine fed a similar purified casein diet supplemented with sulfasuxidine to which a folic acid antagonist is added, develop severe macrocytic anemia, leukopenia, slight thrombocytopenia and hyperplasia of the bone marrow. In the bone marrow there is an increase in immature nucleated red cells which, furthermore, resemble the megaloblasts seen in the marrow of patients with pernicious anemia. This anemia responds rapidly to the administration of pteroylglutamic acid. Purified liver extracts manifest some hemopoietic activity in such animals but the activity is considerably less than that of pteroylglutamic acid.<sup>4,5</sup>

The purpose of the present study was to determine if our original observation could be

confirmed and, if so, whether the anemia so produced differs either morphologically or in its response to therapy, from the anemia produced in swine given a folic acid antagonist.

**Experimental.** A total of 75 weanling Chester-White pigs, 21 to 28 days of age were used. The animals were divided into three groups as follows:

Group	No. pigs	Type casein	Sulfasuxidine†
			%
I	12	Purified*	2
II	42	Crude†	0
III	21	"	2

All animals received the following vitamins: A, D, thiamine, riboflavin, nicotinic acid, pyridoxine, pantothenic acid, choline and inositol. In addition, those in group II were given pteroylglutamic acid, animals in group I were given para-aminobenzoic acid and those in groups II and III were given biotin. The percentage of casein fed is indicated in Tables I and III. Full details of the experimental methods, the composition of the basal diet and the amounts of vitamins administered have been given in previous reports.<sup>1,4,5,9</sup>

**Results.** The hematologic studies in the 12 animals in group I are presented in Table I. Anemia developed in each instance. The anemia was mild (volume of packed red cells 30 to 35 ml/100 ml) in 5 pigs, moderately severe (volume of packed red cells 25 to 30) in 4 pigs, and severe (volume of packed red cells less than 25) in 3 pigs. The anemia was normochromic and in general normocytic although the mean corpuscular volume was greater than 65  $\mu$  in 4 pigs. Differential cell counts on marrow aspirated from the sternum revealed a decrease in the leukocyterythroid ratio with many immature nucleated red cells present. These cells did not differ significantly in their morphologic characteristics from those observed in the marrow of

<sup>2</sup> Cunha, T. J., Colby, R. W., Bustad, L. K., and Bone, J. F., *J. Nutrition*, 1948, **30**, 215.

<sup>3</sup> Welch, A. D., Heinle, R. W., Sharpe, G., George, W. L., and Epstein, M., *Proc. Soc. Exp. Biol. and Med.*, 1947, **65**, 364.

<sup>4</sup> Cartwright, G. E., Fay, J., Tatting, B., and Wintrobe, M. M., *J. Lab. and Clin. Med.*, 1948, **33**, 397.

<sup>5</sup> Cartwright, G. E., Tatting, B., Ashenbrucker, H., and Wintrobe, M. M., *Blood*, 1949, **4**, 301.

<sup>6</sup> Heinle, R. W., Welch, A. D., George, W. L., Epstein, M., and Pritchard, J. A., *J. Lab. and Clin. Med.*, 1947, **32**, 1398.

<sup>7</sup> Welch, A. D., Heinle, R. W., Pritchard, J. A., and Solis, H., *Fed. Proc.*, 1948, **7**, 300.

<sup>8</sup> Heinle, R. W., Welch, A. D., and Pritchard, J. A., *J. Lab. and Clin. Med.*, 1948, **33**, 1647.

\* Either Borden's Labco "vitamin-free" casein or Sheffield's special alcohol extracted casein.<sup>1</sup>

† Sheffield's New Process (Crude) Casein.

‡ Kindly furnished by Sharp and Dohme, Inc., Philadelphia, Pa., through the courtesy of Dr. W. A. Feirer.

<sup>9</sup> Wintrobe, M. M., Miller, J. L., and Lisco, H., *Bull. Johns Hopkins Hosp.*, 1940, **67**, 377.

plicate this measurement, for example, the catheter aperture opens into the stream of blood in the vein and is oppositely situated in the artery. As a result the kinetic energy factor is added to lateral pressure in the first case and subtracted in the second. For this reason one cannot, without velocity measurements or their equivalent, give absolute lateral pressure values. It is possible, however, that with bradycardia the pulmonary vascular pressure gradient direction is actually reversed in diastole. A slow diastolic rise in pulmonary artery pressure seen only in this case would conform with this interpretation. The maximum systolic pulmonary arterial pressure is greatly increased in bradycardia.

In one case the cardiac output was measured by the direct Fick method before, during and immediately after vagal stimulation. The values obtained for the cardiac outputs were 3.2, 2.0 and 2.4 L. per min. in the 3 situations in the order named. The systemic venous pressures rose 2-6 cm H<sub>2</sub>O during the vagal stimulation periods and the mean femoral arterial pressure fell slightly although the pulse pressure increased.

In two experiments with acetylcholine and one with acetyl-beta-methylcholine administered in such doses as to lower the heart rate to a half or less of the control value similar changes in pulmonary vascular pressures were recorded.

The observations recorded herein are entirely in harmony with predictions from classical hemodynamics. They are being reported primarily because they have a bearing on the mechanism of production of pulmonary congestion and edema, in states of bradycardia.

In one respect the effects of acute vagal stimulation appear to differ from the results when comparable bradycardia occurs gradually after increasing the intracranial pressure. In the latter case the pulmonary artery pressure rises, in contrast to the fall reported herein for acute vagal bradycardia. It should be noted that when vagal stimulation was continued for 10 minutes, the bradycardia being maintained, the pulmonary artery pressure rose to values above the control level. The pulmonary venous pressure remains elevated. The mechanism of the delayed rise in pulmonary artery pressure has not been analyzed. It is obvious from the observations reported here that simple bradycardia can account for the elevation in pulmonary venous pressure seen when cardiac slowing results from elevations in intracranial pressure. Since in the latter situation the ensuing pulmonary edema is correlated with the pulmonary venous pressure<sup>3</sup> one can also link the genesis of the edema with bradycardia.

*Summary.* The bradycardia produced by vagal stimulation and by the administration of acetylcholine or acetyl-beta-methylcholine resulted in an elevation of mean pulmonary venous pressure and a small fall in mean pulmonary artery pressure. These observations are discussed in relation to the genesis of pulmonary edema in situations in which bradycardia occurs.

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### 17077. Production of Anemia in Swine Fed Purified Diets and Sulfasuxidine.\*

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In 1946 it was reported from this laboratory<sup>1</sup> that a pig maintained on a diet in which

purified casein (Borden's Labco "vitamin-free") was substituted for crude casein (Sheffield's New Process) and to which 2 per cent

\* Aided by a grant from the United States Public Health Service and the Upjohn Company, Kalamazoo, Mich.

<sup>1</sup> Cartwright, G. E., Wintrobe, M. M., and Humphreys, S., *J. Lab. and Clin. Med.*, 1946, 31, 423.

TABLE III.  
Hematologic Observations in Pigs Receiving Crude Casein.  
(Groups II and III). Mathematical Averages and Ranges.

Dietary casein, %	Group II			Group III		
	No. pigs	VPRC, ml/100 ml	MCV, c.μ.	No. pigs	VPRC, ml/100 ml	MCV, c.μ.
26	10	42.0 (39-47)	53 (51-63)	5	37.3 (31-41)	58 (57-63)
18	10	37.1 (34-41)	56 (50-62)	10	29.7 (25-37)	58 (51-62)
10	22	35.7 (34-40)	55 (49-65)	6	24.6 (21-32)	56 (51-63)

Group II received pteroylglutamic acid and no sulfasuxidine.

Group III received no pteroylglutamic acid but were given sulfasuxidine.

VPRC refers to volume of packed red cells.

MCV refers to mean corpuscular volume.

Figures in parentheses represent range.

acid antagonist, it has also been found that pteroylglutamic acid deficiency develops more rapidly on a diet low in protein.<sup>5</sup>

There were certain dissimilarities between the anemia produced on a crude casein diet and the anemia produced on the diet containing purified casein. No anemia (volume of packed red cells greater than 35 ml/100 ml) was present in 6 (29 per cent) of the pigs fed a crude casein diet. The anemia when it developed was mild (volume of packed red cells, 30 to 35) in 4 pigs (23 per cent), moderately severe (volume of packed red cells, 25 to 30) in 8 (38 per cent), and severe (volume of packed red cells less than 25) in only 2 (10 per cent). Thus the anemia was not produced as consistently, nor was it as severe, as in pigs fed the purified casein. It developed somewhat more slowly and when present, was normocytic. Furthermore, after the development of anemia in the animals fed crude casein in each instance reticulocytosis appeared spontaneously and this was followed by the complete disappearance of the anemia (Fig. 1). The reticulocytosis was gradual in its development and persisted for several weeks, giving a low, flat curve. A sharp peak, such as follows the administration of pteroylglutamic acid (Fig. 1), was not observed. The degree of reticulocytosis varied from 6 to 23 percent and usually reached 10 to 15 per cent.

*Summary.* Pigs maintained on a diet in

which purified casein was substituted for crude casein and to which 2 per cent sulfasuxidine was added developed anemia which responded partially to purified liver extract and completely to pteroylglutamic acid. This anemia resembles that which has been produced in pigs given a folic acid antagonist.

Pigs maintained on a diet containing crude casein and to which 2 per cent sulfasuxidine was added developed less pronounced anemia which disappeared spontaneously.

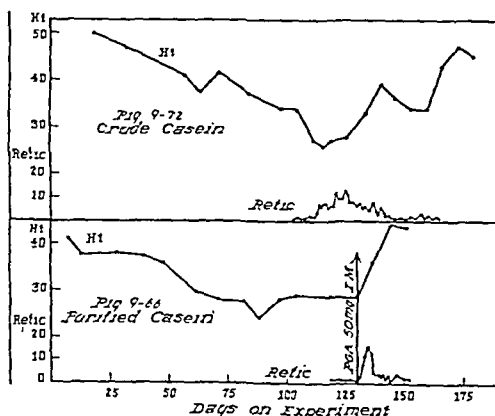


Fig. 1.

Fig 9-72 (Group III) received crude casein (12%). Note the development of anemia and the spontaneous reticulocytosis with relief of the anemia. Fig 9-66 (Group I) received purified casein (12%). Note the persistent anemia and the rapid response with a sharp reticulocyte peak which followed the administration of pteroylglutamic acid.

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TABLE I.  
Hematologic Observations in Pigs Receiving Purified Casein and Sulfasuxidine (Group I).

Pig No.	Casein in diet, %	Day of exper.	RBC millions per c.mm.	Hgb. gm, %	VPRC ml/100 ml	MCV, $\mu$	MCH, $\gamma\gamma$	MCHC, %
9-31	18	135	3.70	6.6	21.0	57	18	31
32	18	206	3.93	9.4	28.0	71	24	34
52	26	136	5.19	10.4	30.5	59	19	33
53	26	85	5.64	9.0	30.0	54	16	33
66	12	130	3.57	8.1	24.5	69	23	33
67	12	130	4.67	10.1	30.0	64	22	34
69	18	130	3.03	6.9	19.5	64	23	35
10-66	26	190	4.68	12.4	34.8	74	26	36
68	26	190	4.15	11.3	32.0	77	27	35
69	26	139	4.52	9.1	28.2	62	20	32
71	26	153	4.60	9.2	28.0	63	21	33
82	26	94	5.13	9.9	29.6	58	19	33

VPRC refers to volume of packed red cells; MCV refers to mean corpuscular volume; MCH refers to mean corpuscular hemoglobin; MCHC refers to mean corpuscular hemoglobin concentration.

TABLE II.  
Response of the Anemia in Group I to Pteroylglutamic Acid (PGA) and Liver Extract Therapy.

Pig No.	Substance	Dose I.M.	Before therapy		After therapy			
			VPRC, ml/100 ml	Reties, %	Retic peak		Vol. pkd. RBC	
					%	day	ml/100 ml	day
9-31	PGA	50 mg	24.5	0.6	5.4	3	37.0	17
32	PGA	50 mg	28.0	0.8	3.6	4	42.5	10
66	PGA	50 mg	24.5	1.0	16.0	5	44.0	14
67	PGA	50 mg	30.0	1.0	11.8	3	40.0	18
69	PGA	50 mg	19.5	0.2	8.0	7	39.5	14
10-66	PGA	20 mg	35.0	2.0	6.0	7	45.5	14
68	Liver extr.*	150 u	32.0	0.8	5.0	2	48.0	28
69	" "	150 u	28.2	2.0	8.0	5	35.0	14
69	PGA	20 mg	31.0	0.8	12.0	5	45.5	21
71	Liver extr.*	75 u	27.0	2.2	6.0	5	37.0	14
71	PGA	20 mg	35.0	1.0	14.0	5	42.0	11

\* Parke, Davis and Company, 15 U.S.P. units per ml.

u refers to U.S.P. units of liver extract.

pigs receiving a crude methyl folic acid antagonist.<sup>4,5</sup> However, in general the changes were not as marked.

The results of therapy with pteroylglutamic acid and with liver extract are presented in Table II. The administration of pteroylglutamic acid resulted in a significant reticulocytosis and a rise in volume of packed red cells to normal. The administration of liver extract was followed by a slight reticulocytosis and a significant although suboptimal rise in volume of packed red cells. In two of the pigs so treated the administration of pteroylglutamic acid resulted in a second reticulocytosis and a further increase in the volume of packed red cells.

The hematologic studies in the animals in groups II and III are presented in Table III where are compared the volumes of packed red cells and the mean corpuscular volumes of pigs fed various proportions of crude casein with and without pteroylglutamic acid and sulfasuxidine. It is clear from these data that, like the animals receiving purified casein (group I, Table I), the inclusion of sulfasuxidine in a pteroylglutamic acid-"free" crude casein diet also resulted in the development of significant anemia (group III). The anemia was comparatively more severe in the pigs fed casein at a 10 per cent level than in those receiving a diet containing 26 per cent casein. In animals given a folic

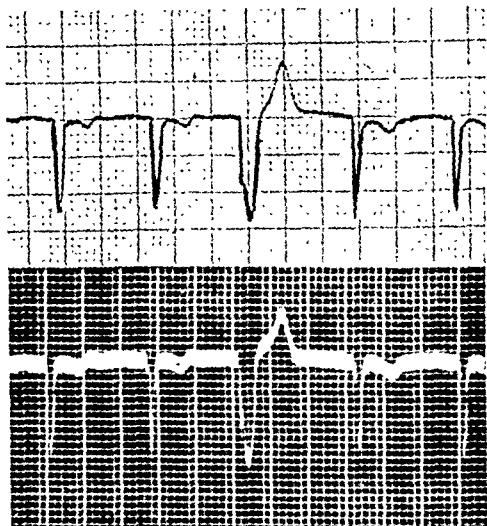


Fig. 2.

Intracavitary potentials recorded simultaneously with a double lumen catheter in right ventricle. Upper tracing was recorded with saline as conductor, lower with usual wire electrode. Form of normal and premature beats are identical. Retouched for publication.

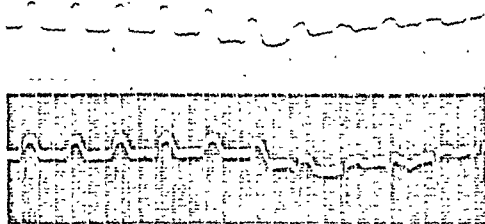


Fig. 3.

Injury in man produced by pressure of tip of double lumen catheter against the endocardium of the right ventricle. As the catheter is withdrawn injury currents disappear. The upper curves were obtained with the saline in one lumen as a conductor. Lower curves were recorded from an indwelling wire which extended to the tip of the second lumen. Note greater ST elevation in the upper record.

differences in contour occurred, due to slight differences in the spatial orientation of the wire electrode tip and the opening of the catheter within the heart. For example, in one patient (Fig. 3) the wire electrode tracing showed definite S-T elevation whereas the open catheter tracing showed more marked S-T elevation, indicating greater currents of injury at its tip, presumably produced by the

open end of the catheter resting squarely against the endocardium.<sup>1</sup> Identical records were obtained when the catheter was withdrawn less than 1 cm. Similarly, simultaneous tracings were also recorded at different levels in the right ventricle, right atrium, and superior vena cava.

Although contour was similar, there was a difference in the amplitude of the complexes when the same standardization was used. Consistently, the tracings were larger when recorded through the usual wire electrode, due to the greater resistance of saline or blood.<sup>2</sup> Since the calculated specific resistance of physiological saline at 25°C is approximately 65 ohm cm<sup>3</sup> and that of copper wire  $1.7 \times 10^{-6}$  ohm cm, this difference in amplitude is quite understandable.

There are certain precautions to be observed if satisfactory intracavity tracings are to be recorded from the single lumen catheter without use of a wire. Alternating current interference is frequently encountered and means should be taken to eliminate it. Small a-c voltage is easily picked up from nearby alternating current circuits or machines. A loose connection or a high resistance at the junction at the arm end of the catheter between the saline and the adapter wire favors a-c disturbance. Extreme care must also be taken to keep the operative field dry. If the arm end of the catheter is in contact with a damp field, the electrical potentials recorded will be a summation of that arm's potential and the intracavitary potential subtended by the tip of the catheter. Thus, we have purposely converted a typical intraventricular electrocardiogram to a left arm unipolar tracing by simply placing the arm end of the catheter electrode connection in contact with a saline-saturated towel on the patient's left arm.

There are certain advantages in the use of this technic. A single lumen catheter can thus be used to obtain blood samples, pressure

<sup>1</sup> Hellerstein, H. K., and Katz, L. N., *Am. Heart J.*, 1948, **36**, 184.

<sup>2</sup> Kaufman, W., and Johnston, F. D., *Am. Heart J.*, 1943, **26**, 42.

<sup>3</sup> MacDougall, F. H., *Physical Chemistry*, New York, Macmillan Co., 1947, pp. 468, 475.

# 17078. Recording of Intracavity Potentials Through a Single-Lumen, Saline Filled Cardiac Catheter.

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The purpose of this report is to present a simple new technic whereby the electrical potential within the human or experimental animal heart can be accurately recorded using a single lumen catheter instead of the usual indwelling wire conductor type of catheter. The column of blood or saline in the catheter acts as the linear conductor to transmit electrical currents arising in the heart.

*Apparatus and methods.* The technic requires a simple electrode attachment consisting of a 2-4 cm length of silver or German silver wire, an adapter to fit into the arm end of the catheter, and an ordinary battery clip. Two models are illustrated (Fig. 1) Both produced identical records. Since a secure junction is essential for tracings free from artefacts, the model with the metal adapter is recommended for general use and especially whenever high intracatheter pressures exist as in retrograde arterial catheterization of the left side of the heart, or in right heart catheterization of patients with pulmonary hypertension.

The catheter is passed in the usual fashion into the cavity of the heart and is kept patent with a slow drip of heparinized physiological

saline or glucose. To obtain intracavity potentials, the above described electrode attachment is connected to the open arm end of the catheter. The exploring terminal of the electrocardiograph is connected to the battery clip of this electrode, and Wilson's central terminal serves as the indifferent electrode. The standardization has been so adjusted that 1 millivolt causes a deflection of 0.8 to 1.2 cm. A radio amplifier type of electrocardiograph is preferred. The electrocardiographic tracing thus obtained represents the potential changes occurring only at the tip of the catheter.

There is a certain amount of admixture of blood with the saline in the tip of the catheter. We have allowed the catheter to fill with blood and compared these tracings with those obtained with a saline flushed catheter. Heparinized saline serves as a more satisfactory conducting medium because of the lesser tendency for blood to clot within the catheter. It is our impression that clotted blood has a greater resistivity, as reflected by a diminution in amplitude of the deflections recorded following only minor clot formation. A study of the comparative electrical conductivity of serum, and liquid and clotted blood is planned in the immediate future.

*Results.* Intracavity potentials have been satisfactorily recorded in 14 patients by this technic. To prove that actual intracavity potential was being recorded, a double lumen electrode catheter was passed into the right ventricle. One lumen contained a wire which extended to the tip, and constitutes the usual cardiac electrode. The electrocardiogram obtained from this cardiac wire electrode was compared with the record simultaneously obtained from the column of blood or saline in the second lumen. In every instance intracavitary potentials were obtained. In general the form of the complexes recorded from each was identical (Fig. 2). Occasionally minor

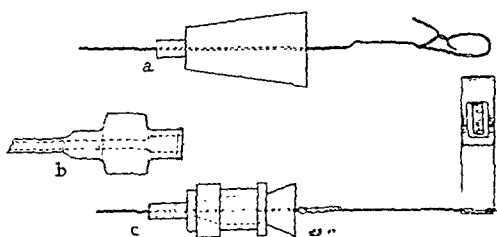


Fig. 1.

(a) Rubber stopper modified to insert into arm end of catheter (b). (c) Is metal syringe adapter with rubber center core. Through each adapter a German silver wire is passed to project 2-4 cm. into the saline filled lumen of the arm end of the catheter. Exploring terminal of ECG is connected to battery clip.

\* Fellow of the National Institute of Health.

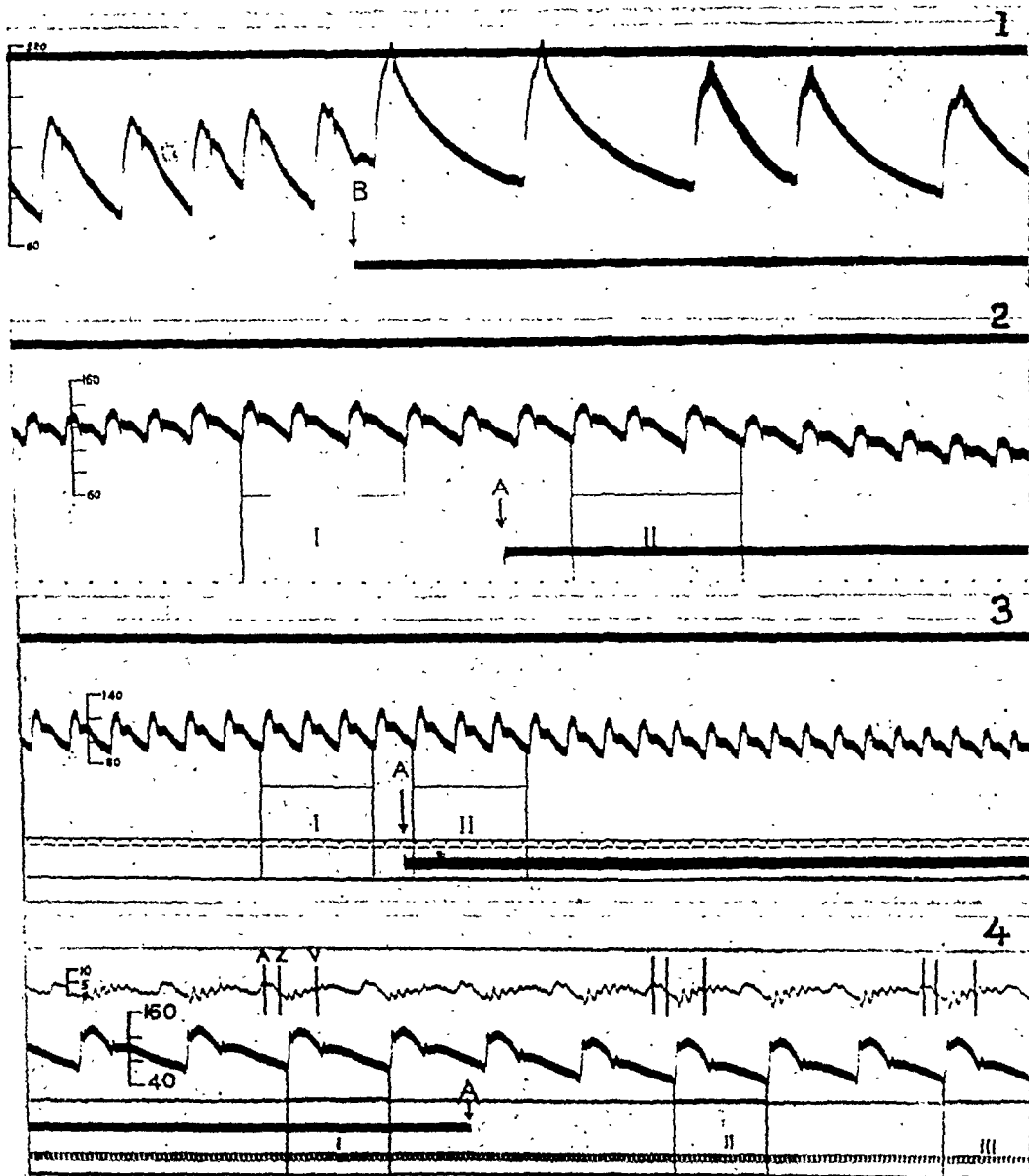


Fig. 1. Aortic pressure pulses showing the effect of closing (B) an A-V shunt on aortic pressure and heart rate. Time, 0.2 sec.

Fig. 2 and 3. Effect of closing an A-V shunt (A) during stabilization of arterial pressure by simultaneous withdrawal of blood. Time, 0.2 sec.

Fig. 4. Effect of opening an A-V shunt (A) on right atrial pressure (upper) and aortic pressure (lower). Time, 0.02 sec.

initial dose of 75 mg per kilo was followed by 15 mg per kilo every hour. Bilateral anastomoses were made between the femoral artery and femoral vein of each side. These

vessels were first cannulated and then joined by rubber tubing. A hemostat was used to open and close the circuit thus produced. Optical manometers of adequate sensitivity



curves, and intracavitary potentials. Some adults and most children have veins too small to allow passage of the usual double lumen catheter electrode.

During the procedure of introducing the catheter, electrocardiographic tracings from the tip of the catheter are valuable in determining the exact anatomical location of the tip. We have found that changes in the contour of the P and QRS complexes may be a more reliable index of the location of the tip of the catheter than either the fluoroscope or the pressure curve, especially in the region of the lower right atrium near the tricuspid valve, or in the coronary sinus.

**Summary.** A new technic is described to obtain intracavitary cardiac potentials through the single lumen saline filled cardiac

catheter without use of the usual copper wire electrode. If the exploring terminal of an electrocardiograph is connected to the open arm end of a column of blood or saline in a catheter which has been introduced into the cavity of the heart, and if the indifferent electrode is connected to a central terminal, the completed curve will represent chiefly the fluctuations of intra-cardiac potential at the tip of the catheter.

Prior to submitting this article for publication we were unaware of the report of Kisch *et al.*, *J. Mt. Sinai Hosp.*, 1948, 15, 257, who described a similar technic although he advocates the introduction of a thin wire into the catheter to avoid a.c. interference.

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### 17079. Reflex Modulation of Heart Rate on Closure and Opening of an A-V Fistula.\*†

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Branham<sup>1</sup> described the well known slowing of the heart rate which ensues upon temporary obliteration of an A-V fistula by digital compression. The reverse phenomenon, *viz.*, increase in heart rate, also takes place when the pressure is released. That the vagus nerves are the efferent pathway of the reflex arc responsible for this reaction has been demonstrated by Weber,<sup>2</sup> Lewis and Drury,<sup>3,4</sup> and

Holman,<sup>5</sup> by use of atropinization techniques. While it is logical to infer that the sinus and aortic nerves constitute the afferent arcs from the arterial side, the possibility that afferents from the venous side may also be concerned has not been excluded. Since closure of an A-V fistula causes a fall, and opening a rise of central venous pressure,<sup>6</sup> such changes in pressure could evoke reflex alterations in heart rate if the Bainbridge reflex is operative. The question as to which afferent pathways are dominantly and subdominantly concerned was therefore resubmitted to experimental study.

**Method.** Dogs varying in weight from 10 to 12 kilos were anesthetized with chloralose because this anesthetic increases the sensitivity of the cardio-inhibitory mechanism, whereby the vagal tone is well maintained. An

\* Supported by a grant from the Life Insurance Medical Research Fund.

† Condensed report of thesis submitted in partial fulfillment of requirements for an M.S. degree, Western Reserve University Medical School, Cleveland, Ohio.

‡ Indian Government Fellow in Physiology.

<sup>1</sup> Branham, H. H., *Internat. J. Surg.*, 1890, 3, 250.

<sup>2</sup> Weber, A., *Munchen med. Wchnschr.*, 1917, 64, 409.

<sup>3</sup> Lewis, T., and Drury, A. N., *Heart*, 1923, 10, 301.

<sup>4</sup> Lewis, T., and Drury, A. N., *Heart*, 1923, 10, 365.

<sup>5</sup> Holman, E., *Ann. Surg.*, 1924, 80, 801.

<sup>6</sup> Heringman, E. C., Davis, H. A., and Rives, J. D., *Proc. Soc. Exp. Biol. and Med.*, 1945, 60, 371.

lishing the existence of such a reflex in a crucial way. Had a material fall of right atrial pressure taken place in these experiments while arterial pressure was kept from rising, no one would have contested the conclusion that the Bainbridge reflex is responsible for the degree of slowing shown in Fig. 2 and 3. Having unwittingly devised an experiment in which neither right atrial nor arterial pressure altered significantly, it becomes apparent that these changes in heart rate must be attributed to still a different factor.

The possibility has been suggested<sup>8,9</sup> that differences in pulse pressure, aside from alterations in mean arterial pressure, may operate to cause reflex alteration in heart rate. However, this factor is also excluded in the curves of Fig. 2 and 3, in which the pulse pressure likewise remains unaltered before the heart rate slows. In short, the slight subsidiary changes in heart rate which are still found upon compression of an A-V fistula

remain undetermined.

**Summary.** An attempt was made to determine whether reflexes other than those from arterial pressor receptors are concerned in modulation of the heart rate when an A-V fistula is closed or opened. In dogs under chloralose anesthesia, bilateral A-V shunts were established between femoral arteries and veins, one of which being so arranged that blood could be drained from the animal from the arterial side during closure of the shunt in an amount sufficient to stabilize arterial pressure at a fairly constant level.

In such experiments it was found that slight slowing of the heart rate still occurs on closing a shunt even when arterial pressure falls a little and the pulse pressure is unaffected. Since supplementary experiments of a similar type failed to reveal significant changes in right atrial pressure, changes in central venous pressure were also excluded. These experiments call attention to the difficulty of establishing the operation of the Bainbridge reflex in a crucial manner.

<sup>8</sup> Bronk, D. W., *Harvey Lec.*, 1934, 29, 245.

<sup>9</sup> McCrea, F. D., and Wiggers, C. J., *Am. J. Physiol.*, 1933, 103, 417.

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## 17080. A Newcastle Disease Virus (NDV) Hemolysin.

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The first observations of a virus hemolysin were those by Morgan, Enders, and Wagley<sup>1</sup> in regard to mumps virus. An hemolysin associated with Newcastle Disease Virus (NDV) has not hitherto been described, although its presence has been observed.<sup>2</sup> The methods employed in the present investigation were modifications of those described by Morgan *et al.*<sup>1</sup>

Dr. Erwin Jungherr kindly supplied the California strain of NDV (no. 11,914) used in all experiments. Infected allantoic and amniotic (AA) fluids were harvested together

36 hours after intra-allantoic inoculation of 10-day-old embryonated eggs with NDV. A single pool of fluids, stored in CO<sub>2</sub> ice, was used throughout. In hemagglutination (HA) tests, 0.5 cc of 0.25% hen erythrocytes was added to an equal amount of each virus dilution. Readings were taken after 1½ hours at room temperature. Two per cent cells were used in hemolysis (HL) tests which were incubated for 2 hours at 37°C. Except for 1 test run at pH 6 (Table I), all dilutions were made in isotonic phosphate buffer pH 7.2.

Some properties of the NDV hemolysin are illustrated in Table I. Hemagglutination and hemolysis tests run on the original AA fluids served as controls. These were of especial

<sup>1</sup> Morgan, H. R., Enders, J. F., and Wagley, P. F., *J. Exp. Med.*, 1948, 88, 503.

<sup>2</sup> Florman, A. L., personal communication.

and calibrated against a mercury manometer under static conditions were used to register aortic pressure pulses. Mean blood pressure was determined by planimetry of 3 successive heart cycles.

*Experiments.* Control reactions were first recorded by simply opening and the closing arterio-venous shunts. It was observed that there was always an increase in heart rate with fall of arterial pressure on opening the shunts, and a lowering of the heart rate with rise of arterial pressure on closing the shunts. Fig. 1 illustrates an experiment in which closing of the shunts at B caused diastolic pressure to rise immediately and the heart rate to slow to a pronounced degree.

In all of these control experiments it was found that the change in heart rate on opening of the shunts took place within 1 to 5 beats (average 1.4 seconds), and maximum slowing developed within 3 beats after closure of the shunts. The fact that this slowing of the heart rate is always preceded by a rise of pressure on closing the shunts naturally suggests that the carotid sinus and aortic nerves constitute the afferent pathways.

To test this hypothesis an effort was made to maintain arterial pressure constant after closure of the shunts. By repeated trials it was found that this could be accomplished in a reasonably satisfactory number of experiments by inserting a Y-tube into one of the A-V anastomoses, through which blood could be withdrawn from the animal at the moment that the venous limb of the Y was closed. By this expedient the animal could be bled from the femoral artery at a rate adjusted to counteract the normal rise in arterial pressure. While it was difficult to maintain an absolutely constant level of mean arterial pressure before and after closure of the shunts in all cases, a sufficient number of records were realized in which no changes or only insignificant alterations in arterial pressure took place. A plot of all experiments in which arterial pressure rose, essentially remained unchanged, or fell, indicated that slowing of the heart rate always accompanied a definite rise in arterial pressure. The change in heart rate on fall of arterial pressure was not so uniform. When

this was less than 5 mm Hg acceleration was not a constant feature. In 4 cases moderate acceleration was observed, and in 6 a definite slowing. Two records free from sinus arrhythmia are reproduced in Fig. 2 and 3. In both of these the change in heart rate is strikingly small as compared to that shown in Fig. 1. Calculation showed that in the curve of Fig. 2 a slight slowing of the heart rate takes place without any rise in arterial pressure. Actual measurement revealed a reduction during period II which would be equivalent to 5 beats per minute as compared to period I, in spite of a fall in mean pressure of 3 mm Hg. In Fig. 3 the slowing is even less, being equivalent to two beats per minute with a fall of mean arterial pressure of only 1 mm Hg.

It is tempting to accept the corollary that the small changes in heart rate which still persist when arterial pressure remains stabilized is occasioned by the Bainbridge reflex. Before this assumption may be accepted, however, it is important to establish that venous pressure is in fact reduced by closure of a shunt. For this reason, several experiments were carried out in "open chest" animals in which right atrial pressure was recorded by an optical manometer. No convincing evidence of a significant change of right atrial pressure was obtained. Fig. 4 shows one of the curves obtained on opening the A-V shunt. It is obvious that right atrial pressure is not significantly affected. Careful measurements of the original curves revealed a rise of pressure equal to 6 mm H<sub>2</sub>O at A, of 8 mm H<sub>2</sub>O at Z, and no alteration at V.

*Discussion.* In a sense it was disappointing to find that the A-V shunts employed in these experiments were apparently not large enough to cause a sufficient change of right atrial pressure to test the operation of the Bainbridge reflex. They do not therefore exclude the possibility that such reflexes may operate when larger shunts exist which apparently do cause an elevation of right atrial pressure.<sup>7</sup> This occurrence was fortunate in another way; it pointed out the difficulty of estab-

<sup>7</sup> Holman, E., and Kolls, A. C., *Arch. Surg.* 1924, 9, 837.

TABLE II.  
Adsorption and Elution of Newcastle Disease Virus (NDV) Hemolysin and Hemagglutinin from Hen Erythrocytes.

Test	Dilutions of NDV											Buffer cont.
	2	8	16	32	64	128	256	512	1024	2048	4096	8192
Original AA fluid		4*	4	4	4	4	4	4	4	4	3	0
III		4	4	3	2	1+	1	±	0	0	0	0
A. Incubation 4°C 1 hr												
Supernate	0	0	0	0	0	0	0	0	0	0	0	0
B. Resuspended RBC from (A)												
Incubation 37°C, 2 hr		0	0	0	0	0	0	0	0	0	0	0
Eluted RBC +		0	0	0	0	0	0	0	0	0	0	0
Fresh NDV												
Eluate and fresh RBC		4	4	4	4	4	4	4	4	4	4	0

\* Figures according to a scale of 0 to 4; denote (IIA) degree of hemagglutination and (III) degree of hemolysis.

position that adsorption and elution serve to break up aggregates of virus and give greater dispersion. Hemolytic activity of the supernate could not be tested, since previous hemolysis resulting from incubation at 37°C rendered it unsuitable.

Inhibition of the NDV hemolysin by specific immune sera is demonstrated in Table III. In these tests the original AA fluids, titrated as in Table I, were used in a dilution of 1:32. Paired sera from a hen experiencing a natural attack of Newcastle disease and a pair of sera from a rabbit immunized with NDV, both gave a 64 fold rise of antibody titer in the inhibition of hemolysin test. Sera of 2 rabbits immunized against influenza virus, one with the PR 8 strain and one with LEE B, and a pair of acute and convalescent phase mumps sera were without specific inhibitory action.

To determine whether the infectivity of the NDV suspension was associated with the hemolytic activity, the AA fluids were treated with 0.2% formalin and incubated over night at 4°C. The hemagglutination and hemolysis tests showed no decrease in titer when formalin treated virus was used. An infectivity titration was carried out in embryonated eggs, using an aliquot of the formalin treated suspension. The formalin treatment lowered the LD<sub>50</sub> end point of the NDV suspension from 10-9.45 to 10-1. Inoculation of controls indicated that 0.2 per cent formalin in broth was not lethal for embryonated eggs.

*Discussion.* Although it is impossible on the basis of present data to state whether the hemolysin associated with NDV is identical with the hemagglutinin or represents a separate factor the behavior of both is more or less parallel in the above experiments. This parallelism is noteworthy in regard to adsorption and elution from red blood cells, the response to heating and to lowering of the pH, as well as in reaction to erythrocytes of different animal species and the inhibitory effect of specific immune serum. Neither activity appears to reflect the infective potency of an NDV suspension as judged by the action of formalin. The NDV hemolysin exhibits points of similarity with the hemolysin associated with mumps virus. However,

TABLE I. Effects of Heat, pH, and Species Differences in Erythrocytes on Hemagglutination and Hemolysis by Newcastle Disease Virus (NDV).

	Test	Dilutions of NDV												Buffer cont.
		8	16	32	64	128	256	512	1024	2048	4096	8192		
Control Titration <i>Effect of heat</i> 51°C 15 min.	HA	4*	4	4	4	4	4	4	4	4	3	0	0	
	HL	4	4	4	3	2	1+	1	±	0	0	0	0	
	HA	4	4	4	4	4	4	4	4	3	0	0	0	
	HL	3	3	3	2+	2	1+	1	±	0	0	0	0	
60°C 8 min.	HA	0	0	0	0	0	0	0	0	0	0	0	0	
	HL	0	0	0	0	0	0	0	0	0	0	0	0	
<i>Effect of pH</i> pH 6	HA	4	4	4	4	4	4	4	4	4	0	0	0	
	HL	4	3	2	2-	1+	1	±	0	0	0	0	0	
(Brown)														
<i>Erythrocytes different species</i> Sheep	HA	4	4	4	4	4	4	4	0	0	0	0	0	
	HL	4	4	3	2	1	0	0	0	0	0	0	0	
	HA	4	4	4	4	4	4	4	0	0	0	0	0	
	HL	4	4	3	2	1	±	0	0	0	0	0	0	
Human "O"	HA	4	4	4	4	4	4	4	0	0	0	0	0	
	HL	4	4	4	4	4	4	4	0	0	0	0	0	
	HA	4	4	4	4	4	4	4	0	0	0	0	0	
	HL	4	4	3	2	1	±	0	0	0	0	0	0	

\* Figures according to a scale ranging from 0 to 4, denote (HA) degree of hemagglutination and (HL) degree of hemolysis.

importance in reading the hemolysin (HL) tests, as the figures given were arrived at by direct comparison with the control tubes used as standards of the degree of hemolysis present. In a 1:8 dilution of the original AA fluid, 100% of the hen erythrocytes were hemolyzed, only cell stroma sedimenting to the bottom of the tube. Heating at 51°C for 15 minutes lowered the hemagglutinin and hemolysin titers only slightly, but both activities were destroyed after heating at 60°C for 8 minutes. The experiments carried out at pH 6 were not wholly satisfactory. For one thing, hen erythrocytes tend to agglutinate spontaneously in the absence of virus as the pH is lowered, a phenomenon regularly observed below pH 5. Secondly, hen hemoglobin is apparently altered at pH 6, as it becomes brown. Readings of the hemolysin were made by adding a small amount of HCl to the control tubes, to render the standards comparable. With these reservations in mind, it would appear the hemagglutination and hemolysis by NDV show some decrease in titer at pH 6. Sheep and human "O" cells gave lower titers in the hemagglutination and hemolysis tests than did hen erythrocytes. Normal AA fluids gave no hemolysis.

The NDV hemolysin can be absorbed and eluted from red blood cells as indicated in Table II. In these experiments 1 cc of NDV-infected AA fluids was added to 5 cc of a 10% suspension of hen erythrocytes and the mixture centrifuged, after incubation for one hour at 4°C. All of the hemolytic and the greater part of the hemagglutinating activity of NDV was removed from the supernatant by adsorption on the erythrocytes as shown under A of Table II. The sedimented erythrocytes plus adsorbed virus were then resuspended in buffer and incubated at 37°C for 2 hours. As indicated under B of Table II the erythrocytes recovered by subsequent centrifugation were refractory to the hemagglutinating and hemolytic activity of a fresh virus suspension. Tests on the supernatant fluid using fresh erythrocytes demonstrated that NDV had eluted, for the hemagglutination titer was even higher than in the original AA fluid. This elevation of hemagglutination titer has been explained by others<sup>3</sup> on a sup-

# 17081. Effect of Pregnancy on the Rat Ovary Transplanted to the Spleen.\*

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The response of the ovary of rats to endogenous hormones, when it is placed in the portal circulation by means of intrasplenic transplantation, differs greatly from that of the ovary that is in its normal position. If the animal is a castrate, the intrasplenic ovary shows a continuous formation of new follicles which luteinize. The corpora lutea do not involute, and from them a neoplasm may ultimately develop.<sup>1,2</sup> If the animal is a unilateral castrate, the intrasplenic ovary atrophies after a period of 24 days, but this atrophic ovary assumes active growth as soon as the normal ovary is removed.<sup>3</sup> It is probable that when one ovary is intact and the other is transplanted to the spleen both are receiving the same hormonal influences through the systemic circulation. The normal ovary shows the compensatory hypertrophy that customarily follows unilateral castration, while the intrasplenic ovary simultaneously is undergoing severe atrophy. That the splenic pulp does not interfere with growth is proved by the appearance of follicles and corpora lutea in the intrasplenic ovary and its progressive enlargement, immediately following the removal of the normal ovary.<sup>3</sup>

The present investigation is a related study on the effect of the endogenous chorionic gonadotrophic hormones of the rat on the normal and on the intrasplenic ovary. Young adult female rats of the Long-Evans strain were employed. The left ovary was removed under ether anaesthesia through a lumbar approach

and cleaned of fat and the tube. The spleen was delivered into the wound, and the left ovary was placed in a pocket made by incising the splenic capsule. After an interval of from 40 to 50 days, the rats during estrus were placed with males, and all became pregnant. Six animals were sacrificed as soon as possible after the litter was delivered; nine additional animals were permitted to live between 9 and 60 days after the termination of pregnancy. Another group of nine animals was ovariectomized as soon as possible after the litter was delivered and subsequently three of these were sacrificed on each of the following days after the birth of the litter: 10, 31, and 62.

Pregnancy had occurred only in the right horn of the uterus of each animal. The litters were composed of normal young which were removed from the mother at birth. In the group ovariectomized post partum, the right ovary was removed through a right lumbar incision, and the spleen was carefully examined for adhesions between it and the parietal abdominal wall or other organs. A complete autopsy was performed on each animal. The tissues were prepared in the usual manner for histologic study and multiple sections at several levels of the transplanted ovary were studied.

The 6 animals that were sacrificed at the termination of pregnancy showed a slight increase in the size of the transplant. Histologic examination revealed that this was due to an increase in the size and number of follicles. Many of the follicles were large and contained an ovum that was compactly surrounded by as many as ten rows of follicular cells. A single cross section through a transplant often contained as many as 10 to 15 follicles of varying size. In one transplant, there was a single small corpus luteum; serial sections of the remaining transplants did not disclose a corpus luteum. The control

\* This investigation was supported in part by a research grant from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service, and in part by a grant from the United States Vitamin Corporation.

<sup>1</sup> Biskind, M. S., and Biskind, G. R., *Proc. Soc. Exp. Biol. and Med.*, 1944, **55**, 176.

<sup>2</sup> Biskind, G. R., and Biskind, M. S., *Am. J. Clin. Path.*, in press.

<sup>3</sup> Biskind, G. R., and Biskind, M. S., *Science*, 1948, **108**, 137.

TABLE III.  
Inhibition of NDV Hemolysis by Specific Immune Serum.

Source of serum	Serum phase	Dilution of NDV								Serum cont.
		16	32	64	128	256	512	1024	2048	
Hen—natural Attack with NDV	Acute	1*	2	3	3	3	3	3	3	0
	Conval.	0	0	0	0	0	1	2	3	0
Rabbit—immunized with NDV	Pre-inoc.	1	2	3	3	3	3	3	3	0
	Immune	0	0	0	0	0	1	2	3	0
Rabbits immunized with influenza virus	PR8-immune	1	2	3	3	3	3	3	3	0
	LEE B. "	1	2	3	3	3	3	3	3	0
Mumps patient	Acute	1	2	3	3	3	3	3	3	0
	Conval.	1	2	3	3	3	3	3	3	0

\* Figures denote degree of hemolysis as compared to control titration. Table II).

in other respects, the NDV hemolysin as demonstrated above differs from the description of the mumps virus hemolysin given by Morgan *et al.*<sup>1</sup> especially as regards the effect of heat and a lowering of the pH. Whether these differences are real or a reflection of the fact that the NDV hemolysin is more potent is not easy to determine. In our experience, it has been difficult to find a mumps virus hemolysin of sufficient potency for comparative tests.

The fact that hemolysins are associated with both NDV and mumps virus, but not with influenza virus,<sup>1</sup> is of interest in indicating a possible affinity of mumps and Newcastle viruses, as first suggested by Burnet<sup>3,4</sup> in his work on "receptor gradients." Recent demonstration of serologic relationships between NDV and mumps virus,<sup>5</sup> evidenced by

the presence of neutralizing and antihemagglutinating antibodies in nearly half of patients convalescent from mumps, lends further support to this hypothesis.

*Summary.* (1) A hemolysin associated with the virus of Newcastle disease is described. (2) The activities of this hemolysin paralleled those of the NDV hemagglutinin in regard to adsorption and elution from hen erythrocytes, inhibition by specific immune serum, inactivation by moderate heating, and in its behavior with erythrocytes of different animal species. (3) A suggestion is made that similarities in behavior between the hemolysin associated with NDV and that associated with mumps virus are further indication of possible relationship between the 2 viral agents.

<sup>3</sup> Enders, J. F., Levens, J. H., and Robbins, F. C., personal communication.

<sup>4</sup> Burnet, F. M., *Australian J. Sci.*, 1945, 8, 81.

<sup>5</sup> Jungherr, E., Luginbuhl, R. E., and Kilham, L., to be published.

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## 17082. By-passing the Right Ventricle.\*

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A comparative analysis of the physiology of pulmonary and systemic pressures of the several classes of vertebrates indicated that the vascular tree of respiratory organs is a circuit of very low resistance.<sup>1</sup> The pulmonary arterial pressure was found to be of the order of 25.10 mm Hg in fish, amphibiae, reptiles, mammals and birds, while the systemic pressures varied according to class and body temperature.<sup>1,2</sup> Consideration of these data suggested that the *vis a tergo* for pumping the venous blood through the lungs might be supplied adequately by the venous pressure. Further, in the presence of a congenital or acquired stenosis of the tricuspid or pulmonary orifice, an atrio-pulmonary shunt by-passing the right ventricle might provide a means for complete oxygenation of the entire venous return. Experiments were done on acute and chronic preparations to demonstrate the feasibility of this procedure.

**Methods and Results.** Acute experiments were carried out on 5 dogs anesthetized with pentobarbital. A cannula was placed in the left atrial appendage and connected with a modified Ludwig Stromuhr. The outflow from the stromuhr was via a cannula tied into the pulmonary artery so that the blood would flow in the direction of the lungs. The pulmonary artery was then tied at its origin. In this way blood was shunted from the right auricle directly to the pulmonary artery without passing through the right ventricle. Pontamine-fast Pink BL (150 mg/kilo) or heparin were used as anticoagulants.

On connecting the stromuhr, blood was seen to flow directly through the glass system. The venous pressure, measured in the right auricle,

was found to be 9 to 14 cm of saline. On tying the origin of the pulmonary artery, the right ventricle ballooned out but the dilation regressed as the experiment continued. The preparations lasted up to one hour.

Chronic experiments were carried out in 5 dogs. Under pentobarbital anesthesia and using artificial respiration via an untracheal catheter, the thorax was opened at the third intercostal space. The pericardium was opened via a longitudinal incision, taking care to avoid the phrenic nerve. The right atrial appendix was clamped by a nontraumatic, specially designed C-shaped forceps.† A second C-forceps was used to occlude about 3 cm of the right aspect of the main pulmonary artery parallel to its trunk. Using 5.0 silk on atraumatic needles, the atrial appendage was brought into approximation and anastomosed to the clamped portion of the pulmonary artery. Minimal bleeding was seen on removal of the C-clamps. The pericardium was closed only partially to prevent pericardial tamponade. The thorax was closed with linen ligatures.

The animals showed prompt recovery from the operative procedure. A loud blowing systolic murmur was heard over the entire thorax, loudest at the apex. Venous pressures were found to be about 7 cm of saline. Fluoroscopy indicated a heart size within normal limits. Dogs with these atrio-pulmonary artery anastomoses have been observed for periods up to two months. They show no notable disability. Systemic arterial blood pressures recorded with the Hamilton manometer were about 180/100 mm Hg.

In one dog, after the anastomosis as described above was completed, the origin of the pulmonary artery was tied off almost completely. This procedure tended to kink the anastomo-

\* Aided by the A. D. Nast Fund for Cardiovascular Research.

† This department is supported in part by the Michael Reese Research Foundation.

<sup>1</sup> Rodbard, S., Brown, F., and Katz, L. N., *Am. Heart J.*, in press.

<sup>2</sup> Rodbard, S., *Science*, 1948, 108, 413.

‡ Machined by Mr. Emil Tiger of the Michael Reese Hospital Medical Research Institute Workshop.



transplants in non-pregnant animals rarely contained as many as 5 minute atrophic follicles.<sup>3</sup>

Animals sacrificed at intervals after the termination of the pregnancy showed that the transplants decreased in size and became atrophic. In 7 instances, they resembled the transplants in the animals with one normally situated and one intrasplenic ovary.<sup>3</sup> The ovarian stroma of each of these groups was composed of clusters of small cells that may have been thecal cells. Small follicles were scattered in the stroma. In 2 transplants small corpora lutea were present.

The animals in the last group had one ovary removed at the termination of pregnancy and subsequently were sacrificed at the intervals mentioned. The intrasplenic ovary grew rapidly and increased in size as early as ten days after the operative procedure. It was composed of developing follicles and corpora lutea. At 31 and 62 days after ovariectomy, some ovaries seemed larger than the corresponding intrasplenic ones described previously.<sup>2</sup> It is apparent that when the normally situated ovary is removed, the hormonal factors originally described are permitted to influence the follicular and luteal growth. In this instance there may be a slightly greater degree of growth of the intrasplenic transplant because of the extent of follicular growth during pregnancy.

The results suggest that in pregnant rats there is a decided difference in the response of the intrasplenic compared to the normal ovary. In the non-pregnant rat, the normal ovary is hypertrophied, the intrasplenic ovary is atrophic. Pregnancy produced the usual large corpora lutea in the hypertrophied normally situated ovary, follicular growth in the intrasplenic ovary of 5 rats, and corpora lutea in 1 rat. Several hypotheses can be adduced to account for these unusual responses of the same gonadal tissues in dif-

ferent sites in the body. The action of the liver in inactivating the estrogenic hormones elaborated by the intrasplenic ovary may play a role. Consideration must be given to the effect of the estrogen secreted into the systemic circulation by the normally placed ovary and its probable limitation of growth on the intrasplenic ovary, either directly or by influencing the hypophysis. In addition, there is an increase in estrogens as well as in progesterone during pregnancy which may act on the intrasplenic ovary. The production of follicular growth only in the intrasplenic ovary in five out of six animals may be due to the increased amounts of chorionic gonadotrophins from the placenta, or to increased hypophyseal stimulation. Further studies to elucidate this unusual effect are being performed.

*Summary.* In 24 rats the left ovary was transplanted to the spleen, and between 40 and 50 days later they were permitted to become pregnant. Six animals were sacrificed at the termination of pregnancy, and in all the intrasplenic ovary showed distinct proliferation of new follicles, and only one of these in addition contained a corpus luteum. Nine animals were sacrificed at intervals up to 60 days post partum, and in these the intrasplenic ovary showed progressive atrophy. In another series, the normal right ovary was removed at the termination of pregnancy and groups of 3 animals were sacrificed 10, 31, and 62 days later. The intrasplenic ovary of this series showed progressive enlargement with the continuous formation of new follicles and corpora lutea, and there was no evidence of involution of the latter. In this experiment, the gonadotrophic hormones of pregnant rats produced primarily follicular activity in the intrasplenic ovary, while simultaneously in the normally situated ovary corpora lutea were formed and maintained.

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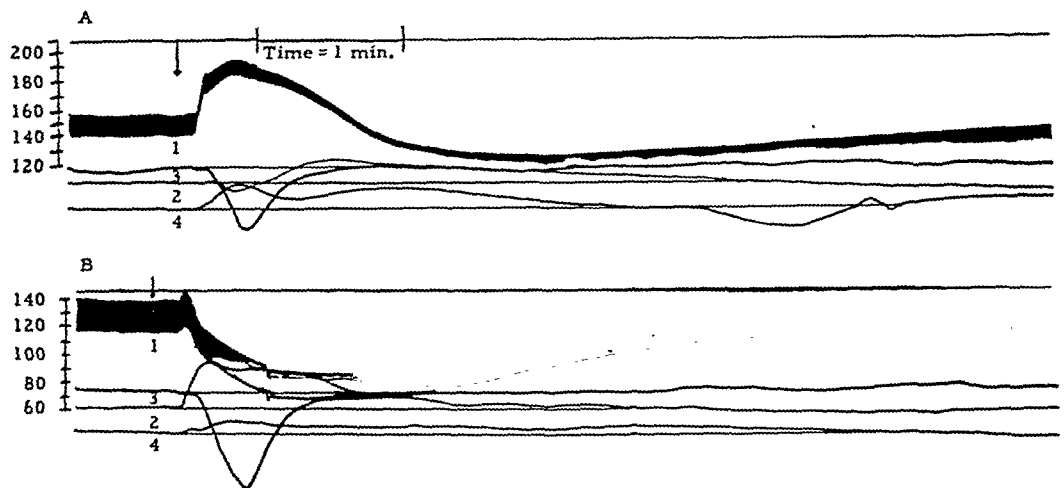


FIG. 1.

A. Cat under Dial anesthesia (70 mg/kg). The effects of 5 µg epinephrine intravenously (injected at arrow) are demonstrated on 1) blood pressure, 2) flow in mesenteric artery, 3) flow in renal artery, and 4) flow in femoral artery. Flow rates were determined with the Rein Thermoströmuhr. B. Same animal 30 min. after 1 mg C-7337 subcutaneously. Note chief effect is "epinephrine reversal." Flow rates qualitatively the same as in A. Time = 1 minute.

In mice the  $LD_{100}$  of epinephrine subcutaneously injected is 7.5 mg. C-7337, 0.03 mg injected subcutaneously half an hour before the epinephrine, permitted a 20% survival; 0.1 mg administered in the same manner protected 40% against the lethal dose of epinephrine; after C 0.3 mg there was 100% survival.

2. The rise of blood pressure after epinephrine (0.1-10 µg) was diminished in cats, dogs and rabbits (Dial anesthesia) by doses of 0.01-0.03 mg C-7337 intravenously or subcutaneously and completely inhibited in cats and dogs by 0.1 mg intravenously or subcutaneously. The typical "epinephrine reversal" was produced in cats and dogs by doses of 0.1-1.0 mg intravenously or subcutaneously (Fig. 1). The epinephrine reversal was also observed in rats and guinea pigs and in the spinal cat. Despite this inversion, the typical effect of epinephrine on blood flow in the renal and femoral arteries remained qualitatively unchanged (Fig. 1).

3. The sustained vasoconstriction produced by constant arterial perfusion of the posterior extremities of the rabbit with epinephrine ( $10^{-7}$ ) was decreased 21% by  $10^{-8}$  C-7337, 50% by  $10^{-7}$  and 90-100% by  $10^{-6}$  (Fig. 2).

4. The contractile action of epinephrine (2

µg) on the nonpregnant rabbit uterus *in vivo* was almost entirely obliterated by 100 µg of C-7337.

5. The relaxing effect of epinephrine ( $5 \times 10^{-7}$ ) on the isolated ileum of the rabbit was diminished approximately 50% by  $5 \times 10^{-7}$  C-7337 and about 65% by  $5 \times 10^{-6}$  C-7337.

6. The minimal doses of C-7337 required to eliminate salivation in the cat were 50 µg for adrenolysis (3 µg epinephrine) and 700 µg for sympatholysis (10 second faradization of cervical sympathetic nerve).

The adrenolytic effect of C-7337 (50 µg) against 3 µg of epinephrine as a sialogogue was complete, but this blocking effect could be partially broken through by 10 or 15 µg of epinephrine.

7. As a rule higher doses of C-7337 were required to produce an adrenolytic and sympatholytic effect upon the nictitating membrane than upon blood pressure and the salivary gland. The retraction of the nictitating membrane produced by epinephrine (10 µg) was almost completely inhibited by 1.0 mg C-7337; after 10 seconds of faradization it was eliminated by 17.7 mg C-7337.

8. Epinephrine hyperglycemia (50 µg) in rabbits was not prevented with doses of 0.15

sis. The right ventricle dilated and irregular heart action was seen for several minutes. A loud systolic murmur was heard post-operatively. The dog was well for 12 hours, but was found dead the next morning. Post mortem examination revealed a patent functioning anastomosis of 1 cm diameter.

**Discussion.** The acute experiments described above demonstrate that the venous pressure may operate as *vis a tergo* to pump at least some of the venous return through the lungs without benefit of right ventricular action. This illustrates anew the remarkably low resistance of the pulmonary circuit. Obstruction to outflow through the pulmonary artery results in a momentary damming of blood in the great veins until the venous pressure rises to levels of about 12 cm saline. A transmission to the venous pressure of some of the pulse from the distended right ventricle continues because of the resulting equivalent of a functional tricuspid regurgitation. However, it is unlikely that this participates in the development of an increased pressure in the right auricle because of the large distensible venous reservoir in free communication with the atrium.

In the chronic experiments with production of the atrio-pulmonary shunt, a functional tricuspid regurgitation is produced because of

the flow of some of the output of the right ventricle via the pulmonary artery anastomosis to the right atrium. The amount of this regurgitation has not been determined. Under normal circumstances the anastomosis results in a flow from ventricle to pulmonary artery to atrium, especially during the heightened pressure of ventricular systole. The possibility must be considered that some blood passes from atrium to pulmonary artery during diastole when the pulmonary diastolic pressure is low, and especially during atrial systole before ventricular contraction occurs. Production of a stenosis or complete occlusion of the pulmonary artery central to the anastomosis forces the flow of blood from atrium directly to the pulmonary artery.

**Summary.** 1. An anastomosis was made between the right atrial appendage and the main pulmonary artery.

2. In acute experiments, the venous pressure was demonstrated to drive venous return through the lungs without benefit of the pumping action of the right ventricle.

3. In chronic experiments, the anastomosis acts similarly to a tricuspid insufficiency. No deleterious effects were seen in the course of two months observations on such dogs.

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## 17083 P. A New Imidazoline Derivative with Marked Adrenolytic Properties.

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Intense interest in efforts to combat the adrenergic predominance associated with various vascular dyscrasias has resulted in the development of numerous agents of diverse chemical structure, including the hydrochloride of 2- [N-p'-tolyl-N-(m'-hydroxyphenyl) - aminomethyl] - imidazoline (C-7337).<sup>1-3</sup>

**Toxicity.** The acute LD<sub>50</sub> values (20 rats per group) are as follows: Intravenous, 75 mg.\* subcutaneous, 275 mg. oral, 1250 mg. A brief report of chronic toxicity studies in dogs has been made.<sup>4</sup>

### Specific Sympathetic Blocking Effects 1.

3 Meier, R., *Farmacoterapia Actual*, 1948, 44, 84.

\* All doses mg or µg/kg intravenously unless stipulated otherwise.

4 Warren, M. R., Woodbury, R. A., and Trapold, J. H., *Fed. Proc.*, 1949, 8, 343.

<sup>1</sup> Marxer, A., and Miescher, K., to be published.

<sup>2</sup> Meier, R., *New York Academy of Sciences*, 1947, in press.

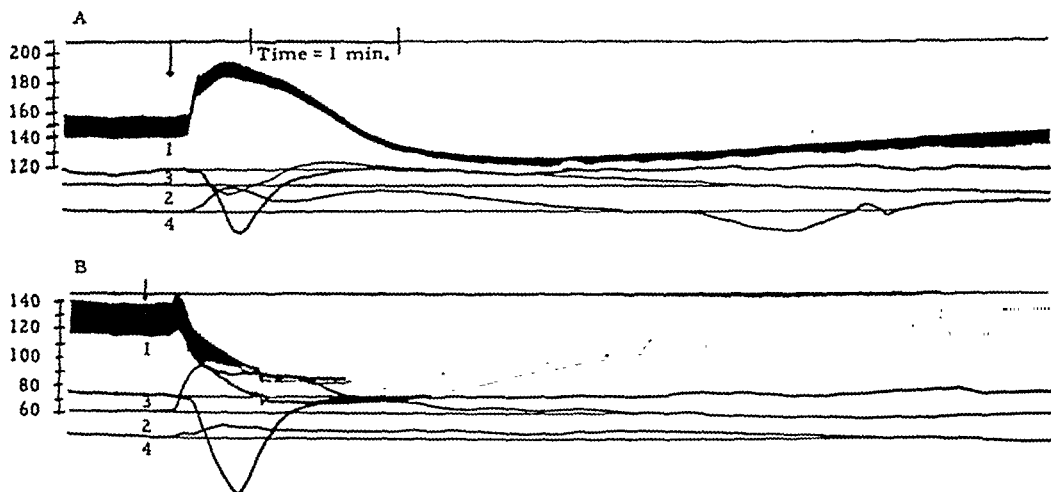


FIG. 1.

A. Cat under Dial anesthesia (70 mg/kg). The effects of 5  $\mu$ g epinephrine intravenously (injected at arrow) are demonstrated on 1) blood pressure, 2) flow in mesenteric artery, 3) flow in renal artery, and 4) flow in femoral artery. Flow rates were determined with the Rein Thermostromuhr. B. Same animal 30 min. after 1 mg C-7337 subcutaneously. Note chief effect is "epinephrine reversal." Flow rates qualitatively the same as in A. Time = 1 minute.

In mice the  $LD_{100}$  of epinephrine subcutaneously injected is 7.5 mg. C-7337, 0.03 mg injected subcutaneously half an hour before the epinephrine, permitted a 20% survival; 0.1 mg administered in the same manner protected 40% against the lethal dose of epinephrine; after C 0.3 mg there was 100% survival.

2. The rise of blood pressure after epinephrine (0.1-10  $\mu$ g) was diminished in cats, dogs and rabbits (Dial anesthesia) by doses of 0.01-0.03 mg C-7337 intravenously or subcutaneously and completely inhibited in cats and dogs by 0.1 mg intravenously or subcutaneously. The typical "epinephrine reversal" was produced in cats and dogs by doses of 0.1-1.0 mg intravenously or subcutaneously (Fig. 1). The epinephrine reversal was also observed in rats and guinea pigs and in the spinal cat. Despite this inversion, the typical effect of epinephrine on blood flow in the renal and femoral arteries remained qualitatively unchanged (Fig. 1).

3. The sustained vasoconstriction produced by constant arterial perfusion of the posterior extremities of the rabbit with epinephrine ( $10^{-7}$ ) was decreased 21% by  $10^{-5}$  C-7337, 50% by  $10^{-7}$  and 90-100% by  $10^{-6}$  (Fig. 2).

4. The contractile action of epinephrine (2

$\mu$ g) on the nonpregnant rabbit uterus *in vivo* was almost entirely obliterated by 100  $\mu$ g of C-7337.

5. The relaxing effect of epinephrine ( $5 \times 10^{-7}$ ) on the isolated ileum of the rabbit was diminished approximately 50% by  $5 \times 10^{-7}$  C-7337 and about 65% by  $5 \times 10^{-6}$  C-7337.

6. The minimal doses of C-7337 required to eliminate salivation in the cat were 50  $\mu$ g for adrenolysis (3  $\mu$ g epinephrine) and 700  $\mu$ g for sympatholysis (10 second faradization of cervical sympathetic nerve).

The adrenolytic effect of C-7337 (50  $\mu$ g) against 3  $\mu$ g of epinephrine as a sialogogue was complete, but this blocking effect could be partially broken through by 10 or 15  $\mu$ g of epinephrine.

7. As a rule higher doses of C-7337 were required to produce an adrenolytic and sympatholytic effect upon the nictitating membrane than upon blood pressure and the salivary gland. The retraction of the nictitating membrane produced by epinephrine (10  $\mu$ g) was almost completely inhibited by 1.0 mg C-7337; after 10 seconds of faradization it was eliminated by 17.7 mg C-7337.

8. Epinephrine hyperglycemia (50  $\mu$ g) in rabbits was not prevented with doses of 0.15

sis. The right ventricle dilated and irregular heart action was seen for several minutes. A loud systolic murmur was heard post-operatively. The dog was well for 12 hours, but was found dead the next morning. Post mortem examination revealed a patent functioning anastomosis of 1 cm diameter.

**Discussion.** The acute experiments described above demonstrate that the venous pressure may operate as *vis a tergo* to pump at least some of the venous return through the lungs without benefit of right ventricular action. This illustrates anew the remarkably low resistance of the pulmonary circuit. Obstruction to outflow through the pulmonary artery results in a momentary damming of blood in the great veins until the venous pressure rises to levels of about 12 cm saline. A transmission to the venous pressure of some of the pulse from the distended right ventricle continues because of the resulting equivalent of a functional tricuspid regurgitation. However, it is unlikely that this participates in the development of an increased pressure in the right auricle because of the large distensible venous reservoir in free communication with the atrium.

In the chronic experiments with production of the atrio-pulmonary shunt, a functional tricuspid regurgitation is produced because of

the flow of some of the output of the right ventricle via the pulmonary artery anastomosis to the right atrium. The amount of this regurgitation has not been determined. Under normal circumstances the anastomosis results in a flow from ventricle to pulmonary artery to atrium, especially during the heightened pressure of ventricular systole. The possibility must be considered that some blood passes from atrium to pulmonary artery during diastole when the pulmonary diastolic pressure is low, and especially during atrial systole before ventricular contraction occurs. Production of a stenosis or complete occlusion of the pulmonary artery central to the anastomosis forces the flow of blood from atrium directly to the pulmonary artery.

**Summary.** 1. An anastomosis was made between the right atrial appendage and the main pulmonary artery.

2. In acute experiments, the venous pressure was demonstrated to drive venous return through the lungs without benefit of the pumping action of the right ventricle.

3. In chronic experiments, the anastomosis acts similarly to a tricuspid insufficiency. No deleterious effects were seen in the course of two months observations on such dogs.

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## 17083 P. A New Imidazoline Derivative with Marked Adrenolytic Properties.

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Intense interest in efforts to combat the adrenergic predominance associated with various vascular dyscrasias has resulted in the development of numerous agents of diverse chemical structure, including the hydrochloride of 2- [N-p'-tolyl-N-(m'-hydroxyphenyl)] - aminomethyl - imidazoline (C-7337).<sup>1-3</sup>

**Toxicity.** The acute LD<sub>50</sub> values (20 rats per group) are as follows: Intravenous, 75 mg,\* subcutaneous, 275 mg, oral, 1250 mg. A brief report of chronic toxicity studies in dogs has been made.<sup>4</sup>

### Specific Sympathetic Blocking Effects 1.

<sup>3</sup> Meier, R., *Farmacoterapia Actual*, 1948, 44, 84.

\* All doses mg or µg/kg intravenously unless stipulated otherwise.

<sup>4</sup> Warren, M. R., Woodbury, R. A., and Trapold, J. H., *Fed. Proc.*, 1949, 8, 343.

<sup>1</sup> Marxer, A., and Miescher, K., to be published.

<sup>2</sup> Meier, R., *New York Academy of Sciences*, 1947, in press.

# 17084. Effects of Desoxypyridoxine and Vitamin B<sub>6</sub> on Development of the Chick Embryo.\*

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The usefulness of metabolite analogs in nutritional and metabolic studies has been demonstrated by a number of workers. These studies have been summarized by several authors.<sup>1-3</sup> It appeared logical, therefore, that vitamin analogs might be used in studies dealing with chemical embryology, and as an approach to this problem, a study of the effects of desoxypyridoxine (2,4-dimethyl-3-hydroxy-5-hydroxymethylpyridine) on the development of the chick embryo was begun. Ott<sup>4</sup> has shown that this analog is markedly inhibitory to growth of chicks on diets which contain amounts of pyridoxine which limit growth, and that its effects are overcome by administration of additional pyridoxine.

While these studies were in progress, Ackermann and Taylor<sup>5</sup> reported the application of this technic to the study of the metabolism of chick embryos. The analog used by these workers, 3-acetylpyridine, was found to inhibit embryonic development and this inhibition could be prevented by nicotinamide. Nicotinic acid and tryptophan were much less active reversing agents.

**Experimental.** The procedure used in injecting the various compounds into the eggs was as follows: With the egg standing on its small end, the large end was swabbed with

ethanol. A small hole was then drilled into the egg through which the compounds were injected. A tuberculin syringe and a No. 26 gauge needle approximately 1.5 cm in length were used for this purpose. The needle was inserted into the egg at such an angle that the solutions injected were deposited in the albumen in close proximity to the yolk. It is possible that occasionally the yolk was pierced by the needle but the data presented show that control injections of water did not result in a significant decrease in hatchability. In the first experiments the compounds were dissolved at concentrations which required injection of 0.2 cc of solution. This amount of water injected into eggs prior to incubation resulted in some decrease in hatchability. One-tenth cc appeared harmless, however, and in further experiments this volume of solution was used. For the injection of higher levels of the compounds at six days of incubation, volumes up to 0.5 cc could be used without harm. After the desired solutions were injected the hole was sealed with collodion and the eggs were immediately set in an incubator.

The eggs were candled at irregular intervals and the infertile eggs and dead embryos were removed. All eggs were broken out and examined to determine fertility and approximate age at death of the embryo. Any gross abnormalities were also recorded.

**Results.** The data of Table I show the effect of the injection of desoxypyridoxine prior to incubation on embryonic development. Approximately 500-1000  $\mu$ g of the analog caused 100% mortality of the embryos. This inhibitory effect of the analog was largely prevented by simultaneous injection of 1.0 mg of pyridoxal hydrochloride. The "toxic" effect of desoxypyridoxine at these levels thus appears to result from its interference with normal utilization of vitamin B<sub>6</sub>, and can be prevented by supplying additional quantities

\* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. We are indebted to Merck and Co., Inc., for gifts of desoxypyridoxine. Reported in part at the Detroit meeting of the American Institute of Nutrition, April, 1949 (*Fed. Proc.*, 1949, **8**, 380).

<sup>1</sup> Roblin, R. O., Jr., *Chem. Rev.*, 1946, **38**, 255.

<sup>2</sup> Woolley, D. W., *Advances in Enzymol.*, 1946, **6**, 129.

<sup>3</sup> Wright, L. D., *J. Am. Dietet. Assn.*, 1947, **23**, 289.

<sup>4</sup> Ott, W. H., *Proc. Soc. Exp. Biol. and Med.*, 1946, **61**, 125.

<sup>5</sup> Ackermann, W. W., and Taylor, A., *Proc. Soc. Exp. Biol. and Med.*, 1948, **67**, 449.

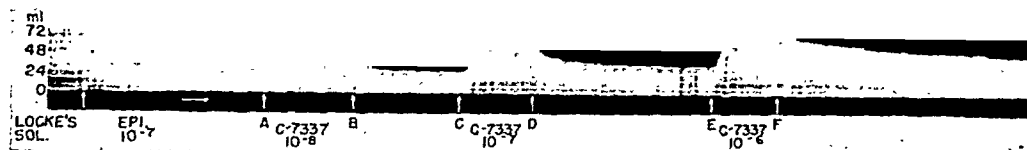


Fig. 2.

Rabbit, hind limbs continuously perfused with Locke's solution containing epinephrine,  $10^{-7}$ . Each vertical line represents an interval of 10 seconds during which the varying rates of flow were registered in milliliters; normal flow was approximately 75 ml per 10 seconds whereas epinephrine,  $10^{-7}$ , reduced this rate to approximately 25 to 30 ml per 10 seconds. From A to B, perfusion with C-7337,  $10^{-8}$ ; from C to D, C-7337,  $10^{-7}$ ; from E to F, C-7337,  $10^{-6}$  concentration. Note the increasing antagonism of epinephrine's vasoconstrictor action following gradually increased concentrations of C-7337.

to 15 mg C-7337 subcutaneously.

9. The adrenolytic effects of adequate amounts of C-7337 (1 to 3 mg) on the vasopressor response to epinephrine ( $2 \mu\text{g}$ ) were observed also after absorption following ileal intubation in the cat and dog.

*Miscellaneous Features.* 1. C-7337 was invariably hypotensive in all species examined (rabbit, cat, dog, rat, guinea pig) and in any dose which produced adrenolytic effects after the conventional, reasonably rapid injection. The hypotensive effect was more marked with increasing dosage and endured—depending on the dose employed—for 2 to 3 hours or more. After intravenous injection the fall in blood pressure was more pronounced than after subcutaneous injection.

2. C-7337 in a concentration of  $10^{-6}$  decreased the coronary flow of the isolated perfused heart of the rabbit 25%, the rate 8% and the amplitude 17%. The corresponding reductions for the feline heart were 30%, 12% and 13%.

3. The isolated vessels of the hind limb of the rabbit showed no reaction to concentrations of C-7337 up to  $10^{-5}$ .

4. No direct myotropic stimulating effect was observed in respect to the bronchi of the rabbit, nictitating membrane of the cat, iso-

lated ilea of the rabbit and guinea pig or intact ileum of the dog. As opposed to the majority of adrenolytic substances, C-7337 has no contractile action upon the isolated uterus of the guinea pig or the uterus *in vivo* of the rabbit.

5. No sialogogic (cholinergic) effect was evident in the cat even with a dose of 27.5 mg of C-7337, whereas faradization of the chorda tympani nerve consistently resulted in salivation.

6. Warburg studies have indicated that C-7337 did not appreciably interfere with feline cardiac respiration until concentrations approaching  $500 \mu\text{g}/\text{ml}$  had been reached. At this concentration there was a 12.5% decrease in cumulative oxygen consumption after 4 hours.<sup>5</sup>

*Conclusions.* The compound 2- [N-p'-tolyl]-N-(m'-hydroxyphenyl)-aminomethyl]-imidazole HCl, (C-7337), is a potent adrenolytic agent but much less active as a sympatholytic drug. It is effective orally.<sup>6</sup>

<sup>5</sup> Herrold, E. A., Cameron, A., Earl, A., Roth, F., Smith, J., Smith, N., Sorensen, E., and Craver, B. N., *Fed. Proc.*, 1949, **8**, 302.

<sup>6</sup> Grimson, K., Chittum, J. R., and Longino, F. H., *Fed. Proc.*, 1949, **8**, 61.

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TABLE III.

Comparative Toxicities of Desoxypyridoxine, Pyridoxal, Pyridoxamine, and Pyridoxine When Injected at 6 Days of Incubation.

Mg of compound injected	% Hatchability†			
	Desoxypyridoxine	Pyridoxal hydrochloride	Pyridoxamine dihydrochloride	Pyridoxine hydrochloride
Uninj. control	100	100	100	100
Inj. control*	70	70	70	70
1.0	80	90	70	100
2.5	70	50	50	90
5.0	0	20	90	80
10.0	0	0	90	10

\* 0.5 cc of water or solutions injected in all cases.

† 10 fertile eggs per group.

TABLE IV.

Comparative Potencies of Pyridoxal, Pyridoxamine, and Pyridoxine in Overcoming the Inhibitory Action of Desoxypyridoxine.

Substance injected*	Amt injected, $\mu$ g							
	0	2	5	10	20	50	70	100 1000
	% hatchability†							
Desoxypyridoxine								0
Desoxypyridoxine, 1000 $\mu$ g								
+ Pyridoxal • HCl		0	0	0	18	30	56	63 47
Pyridoxamine • 2HCl		0	14	27	45	71	67	71 57
Pyridoxine • HCl		0	15	50	74	71	83	62 92

\* 0.1 cc of water or solution injected in all cases. Control groups run at the same time gave the following per cent hatchability: water, 75; 1000  $\mu$ g pyridoxal hydrochloride, 83; 1000  $\mu$ g pyridoxamine dihydrochloride, 62; 1000  $\mu$ g pyridoxine hydrochloride, 60; uninjected, 83.

† All groups contained 12 fertile eggs or over; at critical levels (20 and 50  $\mu$ g of vitamin plus 1000  $\mu$ g of desoxypyridoxine) 23 to 40 fertile eggs were used per group.

desoxypyridoxine which occurs when this compound is injected before the start of incubation.

It has been shown<sup>6</sup> that pyridoxal and pyridoxamine are the predominant forms of vitamin B<sub>6</sub> present in both the egg and the chick; pyridoxine, if present at all, occurs only in relatively small amounts. It was of interest, therefore, to compare the relative activities of these three compounds in counteracting the inhibitory action of desoxypyridoxine. Results of such an experiment are summarized in Table IV. The ratios of vitamin to inhibitor which permitted approximately 50% of the embryos to produce live chicks were for pyridoxal hydrochloride 1:20; for pyridoxamine dihydrochloride 1:50; and for pyridoxine hydrochloride 1:100.

**Discussion.** These experiments show clearly that development of the chick embryo is in-

hibited by the injection of desoxypyridoxine when this is made prior to the start of the incubation period, and that this inhibition is prevented by simultaneous injection of any of the three forms of vitamin B<sub>6</sub>. Vitamin B<sub>6</sub> is an essential catalyst for a variety of reactions involved in protein metabolism, as well as in the synthesis of nicotinic acid from tryptophan. Which of these reactions first fails the embryo because of the blocking action of the inhibitor is not known. Since nicotinic acid may arise from tryptophan in metabolism and since nicotinic acid is synthesized by the chick embryo at an increasing rate as embryonic development progresses,<sup>7</sup> this compound was injected together with desoxypyridoxine to determine whether the inhibitor simply blocks its formation from tryptophan. Neither nicotinic acid nor its amide affected the inhibitory properties of the des-

<sup>6</sup> Rabinowitz, J. C., and Snell, E. E., *J. Biol. Chem.*, 1948, **170**, 1157.

<sup>7</sup> Snell, E. E., and Quarles, E., *J. Nutrition*, 1941, **22**, 483.



TABLE I.  
Effect of Desoxypyridoxine on Hatchability of Eggs.

Substance injected	Amt injected, $\mu$ g	% Hatchability*	
		0.2 cc solutions	0.1 cc solutions
None		74	95
Water (H <sub>2</sub> O)		35	90
Desoxypyridoxine	200	29	
"	500	11	21
"	1000	0	0
+			
Pyridoxal · HCl	1000	13	83

\* Not less than 14 eggs per group.

TABLE II.  
Effect of Injection of Desoxypyridoxine at Different Stages of Incubation of Hatchability.

Time of injection†	% Hatchability*					
	Water	500 $\mu$ g Desoxypyridoxine	Water	500 $\mu$ g Desoxypyridoxine	Water	1000 $\mu$ g Desoxypyridoxine
0 days	88	24	87	14		
68 hr						5
72 "	94	72	86	64	80	4
86 "						53
92 "						79
6 days	93	89	91	91		
9 "	95	88				
12 "	95	89				
15 "	100	95				
Control (uninj.)	95		96		84	

\* 15-23 eggs per group.

† 0.1 cc of water or solution of inhibitor injected in all cases.

of the vitamin. The result demonstrates that vitamin B<sub>6</sub> is highly essential for normal embryonic development. It was observed that most of the embryos died at 2 to 4 days of development.

The effects of injecting desoxypyridoxine at different stages of incubation on embryonic development are shown in Table II. Whereas 500  $\mu$ g of the analog injected prior to setting resulted in approximately 75% mortality of the embryos, a similar quantity injected at 3 or more days of incubation was non-toxic. Injecting 1000  $\mu$ g at 68 or 72 hours of incubation resulted in approximately 95% embryonic mortality. This inhibitory effect was almost immediate since the bulk of the embryos were recorded as dying at 3 or 4 days of development. However, this same amount injected at 86 or 92 hours had little, if any, effect on embryonic development. The inhibitory effects of this analog are thus most marked dur-

ing the first 3 to 4 days of embryonic development; subsequent to this time, the compound has greatly reduced toxicity.

In view of this fact, the toxic level of the analog at 6 days of incubation was determined. In a preliminary experiment, 5 mg of the analog proved almost completely inhibitory at this time, but this inhibition could not be overcome by simultaneous injection of any of the 3 forms of vitamin B<sub>6</sub>. Further experiments, summarized in Table III, indicate that pyridoxal hydrochloride is also toxic at these high levels, while pyridoxine hydrochloride is somewhat less toxic and pyridoxamine dihydrochloride proved non-toxic at the highest level tested (10 mg per egg). Inhibition at this period by these high concentrations of desoxypyridoxine, therefore, is not specifically related to an interference in utilization of vitamin B<sub>6</sub> for essential metabolic purposes, as is the inhibition by smaller amounts of

# 17085. Failure of Human Convalescent and Hyperimmune Monkey Poliomyelitis Serums to Neutralize "Egg Adapted" Lansing C (M) Variant.

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Enright and Schultz<sup>1</sup> reported the successful propagation of the Lansing C (M) strain of poliomyelitis virus in the embryonated hen's egg. Their conclusion was based on the production of typical lesions and paralysis in monkeys with early egg-passage material only. In addition, neutralization was accomplished by anti C (M) rabbit serum, but not by antisera against the murine SK and Theilers GD VII strains, although some cross-reaction was observed with the former. Unreported observations by Schultz<sup>2</sup> have since indicated that the so-called Lansing variant is more closely related to the murine SK strain than to the original Lansing C (M) virus. More recently, Powell and Jamieson<sup>3</sup> have reported the isolation of an apparently similar variant in embryonated eggs. They subsequently proved by mouse protection tests that it was serologically distinct from the original Lansing CM virus and similar to the MM and murine SK strains. Previous unsuccessful or questionable attempts to propagate the Lansing strain to eggs have been reported by Riordan and Sa-Fleitas<sup>4</sup> and Gard.<sup>5</sup>

The egg-adapted variant mentioned above was received from Dr. Edwin W. Schultz in 1948 and readily multiplied in chick embryos.<sup>1</sup> The range of infectivity for mice by the intracerebral route was between  $10^{-5}$  and  $10^{-7}$ . Preparations from the first and subsequent passages made in this laboratory were also infectious for mice by the intraperitoneal, subcutaneous, intravenous, intranasal, and occasionally by the oral route. Seven and a half times

the intracerebral dose was necessary for infection intraperitoneally. The regularity with which paralysis occurred following intraperitoneal injection suggested the possibility of using this means for human serum neutralization tests. For this purpose, 58 specimens of serum from convalescent cases of poliomyelitis were studied. Of these, 33 were taken within 6 months after the onset of the disease, 10 between 6 months and 5 years, and 11 between 5 and 13 years. The time of onset in the other cases was unknown. Immune guinea pig serum was prepared against the Schultz variant for use as a control.

**Methods.** Neutralization tests were performed in the following manner. The virus was prepared so that 0.1 ml contained 50 LD<sub>50</sub> as titrated by the intraperitoneal route. The convalescent serums were diluted either 1:5 or 1:10, and mixed with equal volumes of the virus suspension. Extract broth was used as the diluent in both cases. The mixtures were incubated in a 37°C water-bath for 2 hours, and 0.2 ml inoculated intraperitoneally into 3-week-old Swiss mice. Controls with homologous immune serum, normal guinea pig serum, and in some cases monkey serum were run simultaneously with each series. Six to 8 mice were used for each serum tested. During the course of experiments, three separate lots of egg-passage material were utilized, each being previously standardized for the LD<sub>50</sub> dose. Animals dying within the first 24 hours following injection were discarded; death or paralysis after this period was taken as the end-point and usually occurred during the first week. All experiments were terminated at the end of 2 weeks. Irregular results in the control animals were obtained occasionally. These were found to be due to the use of animals older than four weeks, rather than to deterioration of the virus, which was stored at -6°C.

**Results.** A total of 58 human convalescent

<sup>1</sup> Enright, J. B., and Schultz, E. W., *Proc. Soc. Exp. Biol. and Med.*, 1947, **66**, 541.

<sup>2</sup> Schultz, E. W., personal communication.

<sup>3</sup> Powell, H. M., and Jamieson, W. A., *J. Infect. Diseases*, 1948, **83**, 238.

<sup>4</sup> Riordan, J. T., and Sa-Fleitas, M. J., *J. Immunol.*, 1947, **50**, 263.

<sup>5</sup> Gard, S., *Nature*, 1943, **152**, 660.

oxypyridoxine. Similar negative results were obtained when DL-alanine was injected either alone or with nicotinic acid. DL-alanine was tested since Holden and Snell<sup>8</sup> have shown that D-alanine will replace vitamin B<sub>6</sub> for growth of certain microorganisms.

Umbreit and Waddell<sup>9</sup> have offered an explanation for the mode of action of desoxypyridoxine which may explain our observations that the amount of desoxypyridoxine required to inhibit embryonic growth is increased when injection of the inhibitor is postponed until later in the incubation period. These workers, who used cell preparations containing tyrosine decarboxylase as their test system, showed that desoxypyridoxine exerts its inhibitory effect by being phosphorylated and then competing with pyridoxal phosphate for the apoenzyme. Desoxypyridoxine phosphate was found to be only slightly inhibitory if the pyridoxal phosphate had first been allowed to combine with the tyrosine decarboxylase protein. It is suggested, therefore, that desoxypyridoxine, when injected prior to the start of embryonic development, is phosphorylated and thus is able to compete with pyridoxal phosphate for combination with some newly-formed apoenzyme with which this coenzyme functions, and whose function is necessary for continued development of the embryo. If injection of the inhibitor is delayed beyond about three days, this apoenzyme has already been saturated with pyridoxal phosphate, thus making it impossible for desoxypyridoxine phosphate, even if formed, to exert its inhibitory action.

The toxicity of high levels of desoxypyridoxine when injected at six days of embryonic development was not due to a competitive antivitamin action of the inhibitor since it could not be prevented by any of the 3 forms of vitamin B<sub>6</sub>. Pyridoxal hydrochloride proved to be toxic at similarly high levels while pyridoxine hydrochloride was somewhat less toxic

and pyridoxamine dihydrochloride was non-toxic at the highest level tested.

The role of vitamin B<sub>6</sub> in development of the chick embryo is not readily determined directly, since hens fed diets deficient in this vitamin stop laying before sufficient eggs are obtained to determine its effect on embryonic development.<sup>10</sup> Our experiments show that vitamin B<sub>6</sub> is essential for embryonic development and also that the need for this vitamin becomes critical early in embryonic growth. It is thus possible to use antimetabolites to study problems dealing with the nutrition of the avian embryo which cannot be studied directly.

*Summary.* Desoxypyridoxine (1000  $\mu$ g) injected into eggs prior to the start of the incubation period resulted in 100% mortality of the embryos; this inhibition was prevented by simultaneous injection of any of the three forms of vitamin B<sub>6</sub>. The ratio of vitamin to inhibitor which permitted approximately 50% of the embryos to produce live chicks was 1/20 for pyridoxal, 1/50 for pyridoxamine and 1/100 for pyridoxine.

When injected after 4 or more days of incubation, 1000  $\mu$ g of desoxypyridoxine proved non-toxic. Higher levels injected at 6 days of incubation proved toxic but this toxicity could not be prevented by any of the 3 forms of vitamin B<sub>6</sub>. A possible explanation for this observed variation in toxicity of desoxypyridoxine at different stages of incubation is offered. Pyridoxal hydrochloride and pyridoxine hydrochloride also proved toxic at high levels while pyridoxamine dihydrochloride was not toxic at the highest level tested.

Neither nicotinic acid nor its amide affected the inhibitory action of desoxypyridoxine; negative results were also obtained when DL-alanine was injected alone or with nicotinic acid.

These experiments demonstrate that vitamin B<sub>6</sub> becomes essential for embryonic growth very early in the incubation period.

<sup>8</sup> Holden, J. T., and Snell, E. E., *J. Biol. Chem.*, 1949, **178**, 799.

<sup>9</sup> Umbreit, W. W., and Waddell, J. W., *Proc. Soc. Exp. Biol. and Med.*, 1949, **70**, 293.

<sup>10</sup> Cravens, W. W., Sebesta, E. E., Halpin, J. G., and Hart, E. B., *Poultry Sci.*, 1946, **25**, 80.

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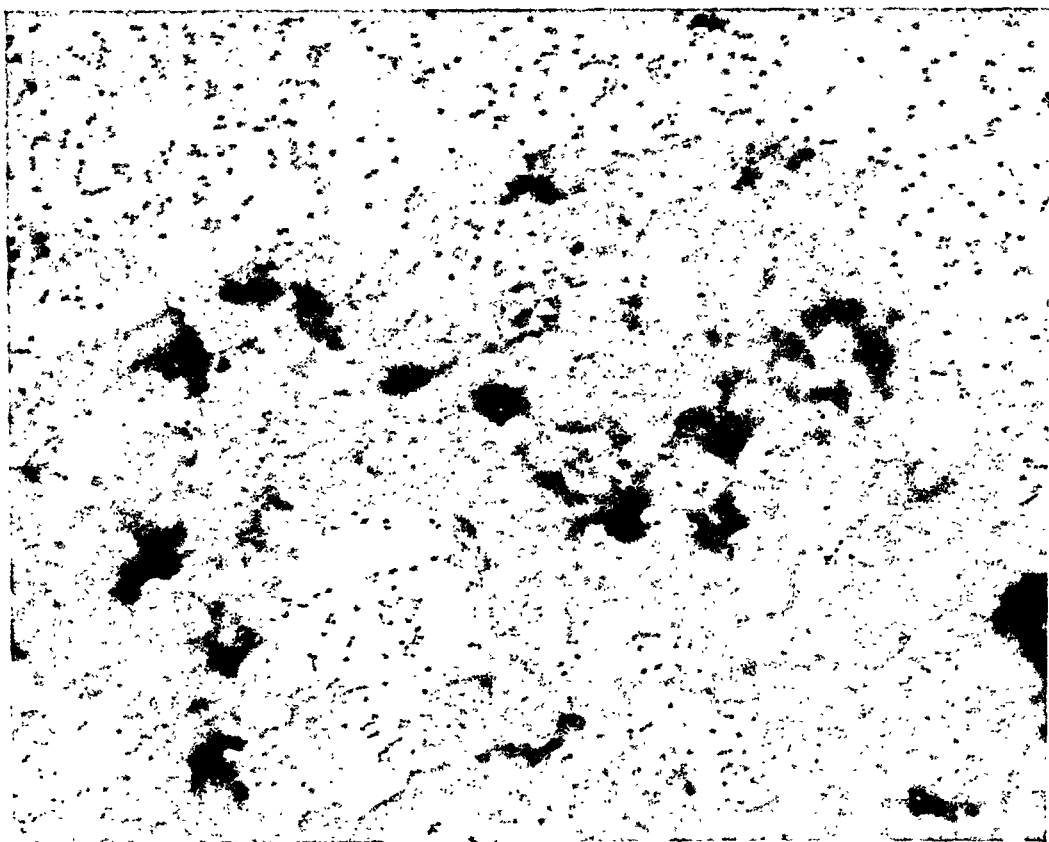


FIG. 1. Preparation of cell destroyed by Eastern equine encephalomyelitis virus.  $\times 15,734$ . Fixed at  $37^{\circ}\text{C}$  with 2% osmic acid vapor for 10 minutes.

days with one transfer in roller tubes<sup>5</sup> in a completely homologous medium composed of chicken plasma and serum and chick embryo extract. Pieces of this tissue were then exposed to a  $10^{-2}$  dilution of the virus. A saline extract of embryos infected with the fourth embryo passage of a standard guinea pig passage of eastern equine encephalomyelitis was used as source of virus. This tissue explant was then placed in a special chamber. This was formed by drilling the center out of a metal plate and sandwiching it between 2 formvar coated glass slides. The medium consisted of 20% chicken serum. Within the next 24 hours of incubation at a temperature of  $37^{\circ}\text{C}$ , the cells had grown out poorly and were already beginning to be destroyed. The

cells were fixed by exposure to osmic acid vapor and were either examined directly or were first coated with chromium to sharpen the image. The fluid taken from the tissue culture chamber taken just before osmic acid fixation of the preparation shows a high titer of infectivity for the chick embryo. (Table I). The titers obtained indicate that considerable multiplication of the virus has taken place. This is indicated by the following figures. An inoculum of chick embryo extract would at the most titer  $10^{-3.5}$ . This inoculum was used in our experiments at a  $10^{-4}$  dilution which was further diluted at least  $10^1$ . The virus, however, was recovered regularly from the tissue culture at  $10^{-6}$  which would be at least 100 times as much virus as was added.

Several things are notable about these pictures: (a) Many particles of uniform

<sup>5</sup> Gey, G. O., *Am. J. Cancer*, 1933, **17**, 752.

<sup>6</sup> Sharp, D. G., Taylor, A. R., Beard, D., and Beard, J. W., *Arch. Path.*, 1943, **36**, 167.

poliomyelitis serums were tested. Of these, 56 showed no neutralization whatsoever. Only 2 gave slight but definite protection. With 50 LD<sub>50</sub> of virus, the 50% end-point with these serums did not exceed 10<sup>-1</sup>. In several experiments complete protection was never obtained with 50 LD<sub>50</sub> of virus, but in two experiments complete protection against 25 LD<sub>50</sub> was provided by a 1:5 dilution of the serums. Of interest is the fact that the serum of one patient was obtained 13 years after the clinical disease, whereas the other was drawn 3 weeks after the onset of paralysis.

Similar tests were carried out using potent hyperimmune monkey serums\* against the Lansing, Kotter (Brunhilde group) and Brockman strains of human poliomyelitis virus. None of these serums protected against the

Schultz variant. Experiments in which 10, 25, and 50 LD<sub>50</sub> of virus were used, together with a 1:5 serum dilution, showed no significant difference between the hyperimmune and pooled normal monkey serums. On the other hand, the "variant" immune guinea pig serum completely protected against 50 LD<sub>50</sub> of MM virus.<sup>†</sup>

*Summary.* The egg-adapted variant originally obtained by Enright and Schultz has not proved to be of value as a means of measuring antibody response in serums from hyperimmune monkeys and convalescent human poliomyelitis cases. The results support the conclusions of Schultz, and of Powell and Jamieson that the egg-adapted variant is serologically related to the MM-SK group of murine encephalomyelitis viruses, rather than to human and monkey-adapted strains.

\* These antisera were obtained through the courtesy of Dr. I. M. Morgan and Dr. J. F. Kessel.

† Obtained from Dr. C. A. Evans.

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## 17086. Electron Microscopy of Tissue Cultures Infected with the Virus of Eastern Equine Encephalomyelitis.\*

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During the past 2 years we have been studying the growth of several viruses in tissue culture with particular emphasis on the use of the electron microscope. The cellular destruction and overwhelming growth of the virus of eastern equine encephalomyelitis are here reported.

Successful electron microscopy of virus infected cells has been limited to studies on "virus induced" tumors<sup>1,2</sup> and has not been applied to small rapidly multiplying viruses.

\* Supported in part by grants-in-aid from the National Institutes of Health, U. S. Public Health Service.

1 Claude, A., Porter, K. R., and Pickels, E. G., *Cancer Research*, 1947, 7, No. 7.

2 Porter, K. R., and Thompson, H. P., *J. Exp. Med.*, 1948, 88, 15.

Our technic is essentially that of Porter<sup>3</sup> except that cells are usually fixed for only ten minutes with osmic acid vapor.<sup>4</sup>

Chick embryo tissue was cultured for 6

TABLE I.  
Equine Encephalomyelitis in Tissue Culture.  
Titration on 10-day Embryos of Fluid Removed  
from Tissue Cultures.

Exp. No.	Original inoculum	Titer of virus in tissue culture fluid
1	10-2	10-5.5
2	10-2	10-6.5+
	10-3	10-6.5+
3	10-4	10-7

3 Porter, K. R., Claude, A., and Fullam, E. F., *J. Exp. Med.*, 1945, 81, 233.

4 Bang, F. B., and Gey, G. O., *Proc. Soc. Exp. Biol. and Med.*, 1948, 69, 86.

obtained after optic nerve section in tadpole and adult frogs;<sup>3,4</sup> and also after eye transplantation when the transplantation is performed near the onset of metamorphosis.<sup>4</sup> This, plus current findings that the optic nerve of teleosts likewise is quite capable of good functional regeneration after severance<sup>5,6</sup> and even, in one case, after eye reimplantation,<sup>6</sup> prompted a further attempt to secure visual recovery after eye transplantation in fishes.

*Experimental.* The goby was selected because of its small size and comparative hardness to surgical trauma and low oxygen tension. The specimens were gathered during February from tide pools on North Bimini. A total of 22 animals, ranging in length from 14 to 29 mm were anesthetized in a 1.5% solution of urethane and operated upon out of water under a dissecting microscope. The left eye was first removed through a dorsal incision in the roof of the orbit. Through a similar incision in the right orbit, the right optic nerve and extraocular muscles were severed. The eyeball was then lifted out of the orbit and cut completely free from all blood vessels and other connections. Any blood clots that formed in the orbit were carefully removed with cleansing tissue before the eye was replaced. In these preliminary experiments only the right eye was reimplanted and it was always replaced in its own orbit and as nearly as possible in its original orientation. These fish, like the frog tadpole, possess a free external cornea which was left intact and which helped to hold the eye in position after its reimplantation.

After the operation the fish were placed in an aquarium with running sea water in a darkroom on the supposition that retinal metabolism might be somewhat reduced in darkness and that this might be beneficial during the early post-operative period while circulation was being reestablished. Beginning on the 6th day after operation the darkroom was lighted for 10 to 20 minutes each day for

feeding the fish and for extraneous purposes. On the 21st day the fish were shifted into two aquaria in the light with standing sea water that was changed every 4 days.

When the animals were examined on the 9th day after operation, the external features of the eye appeared quite normal and eye movements were present in several cases, but there was no sign of vision at this time. When tested again on the 27th day, 2 cases displayed unmistakable evidence of visual recovery. They rose off the bottom and swam directly upward approximately 100 mm toward a small piece of bait less than 4 mm in diameter dangled on the end of a fine silk thread. By the 37th day, 7 of the 16 fish which were still alive had come to perform regularly in this manner. Similar responses were never observed in blind gobies. The accuracy with which those with vision were able to locate small stationary lures indicated that the regenerated fibers had restored their central connections in an orderly manner just as occurs after simple optic nerve section.<sup>7</sup> In control tests in which the bait was presented outside the glass walls of the aquarium all 7 cases responded in typical fashion attempting to reach the lure through the glass. Those which recovered vision measured in total length 16, 16, 18, 22, 24, 25, and 29 mm respectively.

Following anesthetization with urethane on the 38th day, all the heads were fixed in Bouin's solution and were later prepared for microscopic study by a modification of the Bodian Protargol method.<sup>7</sup> Patches of retinal degeneration, particularly in the ganglion cell and inner plexiform layers, were found in all cases but were especially marked in those that failed to recover vision. At some points all retinal layers had disintegrated. In those that recovered vision, a large regenerated optic nerve was present, the fibers of which were easily traceable into the optic tectum of the mesencephalon. Similar regenerated optic nerves were also found in 2 of the cases in which visual feeding reactions had not yet appeared at the time of sacrifice.

<sup>3</sup> Sperry, R., *J. Neurophysiol.*, 1944, **7**, 57.

<sup>4</sup> Sperry, R., *J. Neurophysiol.*, 1945, **8**, 15.

<sup>5</sup> Sperry, R., *Physiol. Zool.*, 1948, **21**, 351.

<sup>6</sup> Rasquin, P., *Physiol. Zool.*, 1949, **22**, 131.

<sup>7</sup> Sperry, R., and Miner, N., *J. Comp. Neurol.*,

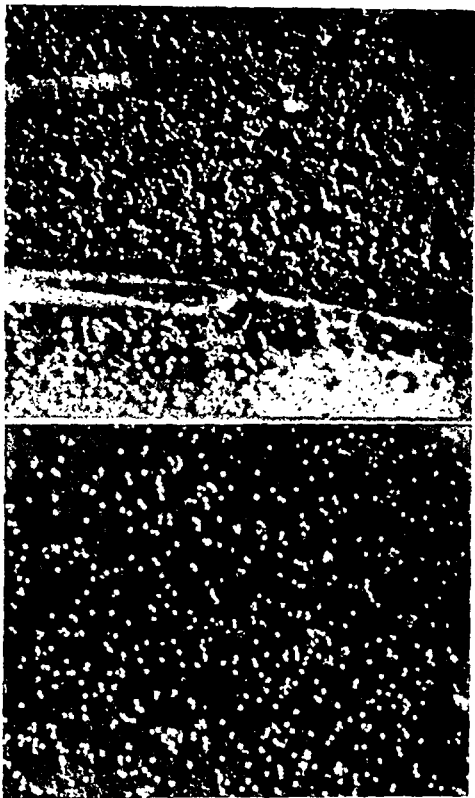


FIG. 2. (top). Chromium shadowed preparation of the same slide. Individual particles may be seen breaking off cell at bottom.  $\times 10,300$ .

FIG. 3. (bottom). Area near a cell. Virus has been scattered over formvar screen. Dividing forms seen throughout.

size similar to those described in purified preparations<sup>6</sup> are associated with destroyed cells and have spilled out into the surrounding areas. The extensive cellular destruction is similar to that produced by infection of the embryo.<sup>7</sup> Such particles have not been seen in similar preparations of cells infected with the viruses of Newcastle disease of chickens, influenza, or mumps. (b) Examination of the pictures shows numerous pairs of particles with occasional chains and small clumps. The general appearance is much like that of a smear of ordinary diplococci. Further, it is relatively easy both in the shadowed and unshadowed pictures to trace a series of stages in the division of the pairs which suggests that multiplication may take place by simple binary fission. (c) These results make it probable that some viruses may be studied by electron microscopy of tissue culture preparations without previous purification from tissue extracts.

<sup>7</sup> Bang, F. B., *J. Exp. Med.*, 1943, **77**, 337.

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## 17087 P. Reimplantation of Eyes in Fishes (*Bathygobius soporator*) with Recovery of Vision.\*

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The early demonstration by Matthey,<sup>1</sup> that the grafted adult urodele eye is capable of recovering visual function has since been extensively confirmed.<sup>2</sup> With regard to fishes and the anuran amphibians, on the

other hand, repeated failure of numerous attempts by various investigators to obtain visual recovery after eye transplantation, even in young tadpole stages, has given rise to the impression that the optic nerve of the grafted eye in these forms lacks the power for functional regeneration.<sup>2</sup>

Recently, however, the writer was able to show that good visual recovery can be

\* The experiments were conducted at the Lerner Marine Laboratory of the American Museum of Natural History, at Bimini, British West Indies. They were aided by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

<sup>1</sup> Matthey, R., *Compt. rend. Soc. Biol.*, 1926, **94**, 4.

<sup>2</sup> Stone, L. S., *Trans. N. Y. Acad. Sci.*, 1941, **3**, 208.

TABLE I.  
Blood Fibrinogen and Adrenal and Thymus Weights During Adaptation to Cold.

No. of animals	Body wt		Time of exposure	Adrenal wt, mg	Thymus wt, mg	Plasma fibrinogen, mg/100 ml
	Initial	Final				
8*	145 $\pm$ 10.5†	—	0	27.7 $\pm$ 1.20‡	271 $\pm$ 18.0‡	252 $\pm$ 12.6‡
7	147 $\pm$ 8.9	138 $\pm$ 8.2	0	34.3 $\pm$ 1.94	163 $\pm$ 9.1	257 $\pm$ 9.3
8	146 $\pm$ 9.7	127 $\pm$ 10.3	10 hr	36.1 $\pm$ 1.50	156 $\pm$ 16.8	242 $\pm$ 15.3
7	148 $\pm$ 11.4	129 $\pm$ 9.5	1 day	35.4 $\pm$ 1.18	117 $\pm$ 9.3	191 $\pm$ 5.9
8	149 $\pm$ 12.7	127 $\pm$ 13.2	2 "	39.4 $\pm$ 1.26	108 $\pm$ 3.9	206 $\pm$ 11.1
8	148 $\pm$ 11.7	122 $\pm$ 9.0	5 "	37.7 $\pm$ 1.86	67 $\pm$ 5.2	208 $\pm$ 7.6
8	149 $\pm$ 10.0	128 $\pm$ 12.0	10 "	42.3 $\pm$ 1.95	91 $\pm$ 7.9	207 $\pm$ 6.5
8	148 $\pm$ 11.5	138 $\pm$ 16.3	21 "	43.4 $\pm$ 3.87	90 $\pm$ 14.2	208 $\pm$ 13.4

\* Not fasted before fibrinogen determination.

† Standard deviations.

‡ Standard errors.

**Results.** Table I summarizes our results. The changes in blood fibrinogen and adrenal and thymus weights during the experimental period are also shown in Fig. 1. The values at zero time are those of the 24 hour fasted controls. The blood plasma fibrinogen decreased sharply from the control value of 257 mg to 191 mg per 100 ml in the first 24 hours. At the end of 48 hours the fibrinogen increased to 206 mg, remaining at this level until the end of the experimental period of 21 days. As can be seen from the table, fasting alone did not influence the blood fibrinogen concentration.

The weight of the adrenals increased rapidly in the first 48 hours and at a slower rate for the remainder of the cold exposure period. The thymus weights show a decrease, which was most marked at the end of the fifth day of exposure to cold, continuing approximately at the same level till the end of the experiment. The weights of these organs were used as an index showing the efficacy of the alarming stimulus. The changes observed are those constantly present in the stages of alarm and resistance of the "general adaptation syndrome".

**Discussion.** It seems apparent that the plasma fibrinogen does not respond in a constant manner to different types of stress as other constituents of body fluids do. In all the previously mentioned instances of blood fibrinogen increase the common feature was the tissue damage, which is known to be a stimulus for fibrinogen production. In this connection, it is noteworthy that the presence

of a fibrinogen-production-stimulating substance in a turpentine-induced abscess in dogs has also been reported.<sup>7</sup>

In the present experiment tissue damage was not excluded. Apart from the intense catabolism indicated by the marked loss of weight, most of the animals developed severe gastric ulcers when exposed to cold and fast-

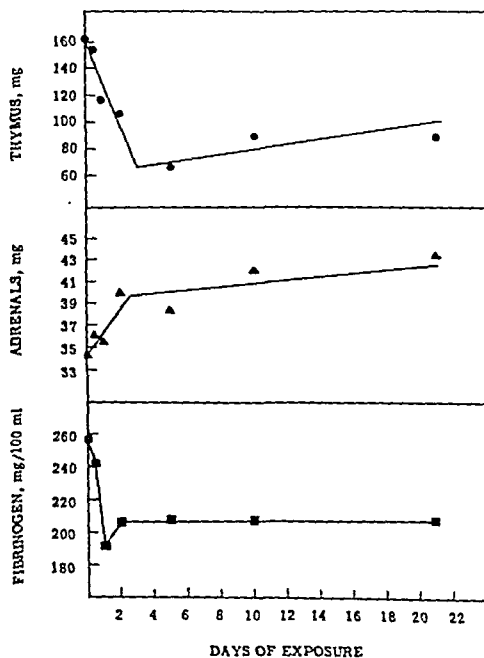


FIG. 1.  
Blood fibrinogen, adrenal and thymus weights during adaptation to cold.

<sup>7</sup> Homburger, F., *J. Clin. Invest.*, 1945, **24**, 43.



## 17088. Influence of Cold on Blood Fibrinogen Concentration.\*

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Changes of several chemical constituents of the blood are known to follow the same pattern under diverse conditions of non-specific stress. These biochemical changes have therefore been considered to be manifestations of the general adaptation syndrome. A survey of the literature suggests that perhaps variations in blood fibrinogen belong to this same group. Thus an increase in plasma fibrinogen has been reported in many non-specific stress conditions, such as infectious diseases<sup>2</sup> and a wide variety of chemical and physical injuries.<sup>3,4,5</sup>

On the other hand, a decrease in plasma fibrinogen appears to be almost always associated with specific disturbances directly related to its production, such as dietary deficiencies<sup>2</sup> or extensive liver damage.<sup>5</sup>

We therefore decided to study the blood fibrinogen changes occurring in rats exposed to cold, during the stages of alarm and resistance of the "general adaptation syndrome."

**Material and Methods.** Eight groups of 8 female piebald rats, weighing 130-165 g (average 145 g) were used in this experiment. All animals were put into the cold room (2-5°C) in individual cages, except 2 groups kept at room temperature as controls. All groups were maintained throughout on Purina Fox Chow, with tap water *ad libitum*. The 6 groups of rats were removed from the cold room, bled and killed, after 10, 24, 48 hours and 5, 10, 21 days respectively. During the last 24 hours of exposure all animals were fasted, but water was allowed. Blood samples were taken by carotid puncture under ether

anesthesia. Blood coagulation was prevented by the use of silicone-coated syringes and needles, the samples being added to tubes containing 3 mg of potassium oxalate per ml of blood.

At the end of the experiment, one group of control rats was bled and killed without fasting, while the other group was fasted for 24 hours before bleeding.

At autopsy all the animals were carefully inspected for any detectable infection. The adrenals and thymus were removed, fixed in formalin and weighed.

The plasma fibrinogen was determined by a modification of the method of Campbell and Hanna<sup>6</sup> with direct nesslerization. In order to avoid the digestion in centrifuge tubes which frequently causes loss of material by bumping, we modified it as follows: the precipitated fibrinogen was quantitatively transferred with distilled water to a Folin 50 ml graduated digestion tube. The tube was then placed in an oven at 90-100°C overnight in order to evaporate the water. The following day the dry residue was digested with 1 ml of the undiluted digestion mixture. Decoloration frequently occurs following nesslerization due to the presence of an inert precipitate formed during digestion. This was prevented by diluting the digest exactly to 50 ml with distilled water, centrifuging the solution and pipetting 25 ml of the clear supernatant. The nesslerization was then performed in a 50 ml cylinder. To avoid the turbidity which occasionally occurs during the nesslerization, the diluted digest was made up to 35 ml with distilled water and the cylinder immersed in a cold bath for several minutes. Then 15 ml of the Nessler's reagent (Folin's formula) was quickly added, mixed immediately by inversion and the cylinder maintained in the water bath until read in the colorimeter. All determinations were made in duplicate.

\* This investigation was supported by a research grant from the Commonwealth Fund.

<sup>1</sup> Selye, H., *J. Clin. Endocrinology*, 1946, **6**, 118.

<sup>2</sup> Ham, T., and Curtis, F. C., *Medicina*, 1938, **17**, 413.

<sup>3</sup> Chanutin, A., Hortenstine, J. C., Cole, W. S., and Ludewig, S., *J. Biol. Chem.*, 1938, **123**, 247.

<sup>4</sup> Chanutin, A., and Ludewig, S., *J. Biol. Chem.*, 1947, **167**, 313.

<sup>5</sup> Foster, D. P., and Whipple, G. H., *Am. J. Physiol.*, 1922, **58**, 407.

<sup>6</sup> Campbell, W. R., and Hanna, M. I., *J. Biol. Chem.*, 1937, **119**, 15.

TABLE I.

Fraction No.	Description of fraction	Inhibitory activity		Toxicity	
		Total No. of eggs	Amt necessary for 50% inhibition, mg per egg	Total No. of eggs	Amt necessary for 50% mortality, mg per egg
1	Hot water extract	71	0.91		
2	Alcohol soluble	43	2.00	25	7.0
3	Alcohol insoluble	27	0.66		
4	Ppt. at 64% alcohol	44	1.59		
5	Ppt. at 65 to 80% alcohol	66	0.84		
6	Ppt. at 81 to 88% alcohol	79	0.41	32	6.6
7	Soluble at 88% alcohol	36	0.92		
8	Ppt. at pH 2-3	29	0.46		
9	Soluble at pH 2-3	21	1.20		
10	Tannic acid, USP	112	0.22	32	1.6

the most active tea fraction, that precipitated at 81 to 88% alcohol (Fraction 6).

Because of its greater activity fraction 6 was chosen for more detailed study. Repeated precipitation with 88% alcohol at pH 5-6 resulted in no significant change in activity. *In vitro* tests, carried out as previously described,<sup>1</sup> show that as little as 25  $\mu$ g per cc will inactivate a significant amount of virus. Two mg of fraction 6 per egg inhibit multiplication when given as long as 2 hours before or 2 hours after inoculation of virus. Injection of 10 mg into the yolk sac 2 hours before inoculation of virus into the allantoic sac does not inhibit multiplication.

**Discussion.** Obviously, attempts at purification have been crude, and probably the fractions prepared are mixtures of uncertain composition. Some fractions should contain tannic acid as an appreciable amount is present in tea.<sup>2</sup> Furthermore, as tannic acid is said to be soluble in alcohol, one would expect any present to be concentrated in the alcohol soluble fractions (No. 2 and 7). If inhibitory activity were due to tannic acid the alcohol insoluble fraction (No. 3) and that precipitated at a concentration of 81 to 88% alcohol (No. 6) should show relatively little activity. However, the reverse is true, these fractions being among the most active ones obtained. These differences in solubility may be more apparent than real, depending upon other variables such as pH, which have not been investigated fully. Common tests for

tannic acid, such as those employing ferric salts and soluble protein, which are notoriously non-specific, have failed to differentiate clearly the various fractions from each other and from tannic acid. The difficulty of deciding whether the active principle is or is not tannic acid or one of the tannins is further emphasized by the fact that the different natural tannins exhibit differences in their chemical reactions and behavior with solvents.<sup>2</sup> Furthermore, even the "purified" or "crystalline" preparations of tannic acid are mixtures, as it is well recognized that they are contaminated with various impurities.<sup>3</sup> Although the effect of fraction 6 on influenza virus is similar to that of tannic acid, the most significant difference may be a biological one, namely, that the former, although only one-half as active as tannic acid, is some four times less toxic.

**Summary.** Extracts of tea have yielded an unidentified fraction, similar to but less toxic than tannic acid USP, which inhibits the multiplication of influenza virus in embryonated eggs.

It is a pleasure to acknowledge the technical assistance of Miss Ann Holloway.

<sup>2</sup> Nierenstein, M., *Tannins*, in *Allen's Commercial Organic Analysis*, Vol. V, 5th edition, P. Blakiston's Son and Co., Philadelphia, 1927.

<sup>3</sup> Nierenstein, M., *The Natural Organic Tannins*, J. and A. Churchill, Ltd., London, 1934.

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ing. The incidence of these ulcers was greatest at 24 hours, which coincides with the most pronounced fall in fibrinogen values. Cold, therefore, could have a specific action of depressing the fibrinogen production. It is equally possible that the plasma fibrinogen fall represents only another particular instance of the generalized protein catabolism during exposure to cold. Thus, an increased catabolism of fibrinogen, if unaccompanied by a corresponding increase in its production, ap-

pears to result in a fall of its concentration in the plasma.

*Summary.* In rats fasted for 24 hours before bleeding, exposure to cold causes a fall in the plasma fibrinogen concentration. The fall is maximal at the end of the first 24 hours and is followed by a slight rise to a subnormal level. This low level is maintained for as long as 21 days after beginning of cold treatment.

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### 17089 P. Inhibition of Multiplication of Influenza Virus by Extracts of Tea.\*

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It was reported<sup>1</sup> previously that tannic acid inhibits the multiplication of influenza A virus in embryonated eggs. The tannic acid used was tannic acid, USP, a commercial sample, presumably extracted from nutgalls. During the course of studies designed to test the inhibitory effect of tannic acid obtained from other sources, attempts were made to extract a suitable preparation of tannic acid from tea. Preparations having certain of the characteristics of tannic acid and showing inhibitory activity have been obtained; in addition, other preparations differing, in some respects, from tannic acid and showing somewhat greater inhibitory activity than the "tannic acid" fractions also have been extracted from tea.

*Materials and methods.* The tea employed was a commercial sample of black tea. The PR8 strain of influenza A was used exclusively. Materials to be tested were dissolved in water, autoclaved, and neutralized with sodium hydroxide. The specified amount, in a volume of 0.5 cc, was injected into the allantoic sac of 10-day embryonated eggs and after an interval of one-half hour approxi-

mately 100 ID<sub>50</sub> of virus were inoculated by the same route. Inhibition of multiplication was considered to have occurred if, after 48 hours incubation, allantoic fluid from eggs so-treated, did not agglutinate chicken erythrocytes.

*Experimental.* A desiccated hot water extract of tea was prepared and fractionated by the following methods: (1) by solubility or insolubility in absolute ethyl alcohol, (2) by fractional precipitation from aqueous solution with various concentrations of ethyl alcohol, (3) by precipitation from aqueous solution on addition of sufficient dilute HCl to reduce the pH to 2-3. Fractions thus obtained were desiccated and injected into groups of embryonated eggs, in amounts increasing by two-fold increments, one-half hour before inoculation of virus. The amount of each fraction per egg necessary to inhibit virus multiplication in 50 per cent of the eggs was then determined. In some instances, toxicities in terms of LD<sub>50</sub> also were determined.

Inspection of Table I reveals that all fractions showed some inhibitory activity. Moreover, activity may be concentrated by removal of alcohol soluble materials, by precipitation at 88 per cent alcohol, and by precipitation at pH 2-3. Tannic acid USP is approximately twice as active but four times more toxic than

\* Aided by a grant from the United States Public Health Service.

<sup>1</sup> Green, R. H., *Proc. Soc. Exp. Biol. and Med.*, 1948, 67, 483.

TABLE I.  
 Effect of *l*-Epinephrine and *l*-Arterenol on Eggwhite Edema in Rats.

Drug used	Dose, $\mu\text{g/kg}$	No. indiv. exp.	No. rats used	Avg % incidence of edema	Avg degree of edema in affected rats
None	0	16	94	90%	2.5+
Epinephrine subcutaneous	5	2	12	85	4
" "	25-30	5	49	95	3
" "	35-40	5	32	90	3
" "	50-55	8	64	95	3.5
" "	60-75	3	21	65	3.5
" "	100	2	11	10	trace
" "	140-150	2	10	40	"
" "	200	1	5	20	"
" "	275	1	5	0	0
" "	550	1	5	0	0
Epinephrine intramuscular	25	1	8	100	3.0
" "	50	2	14	50	1.5
" "	75	1	6	35	2.5
" "	100	2	14	10	trace
" "	200	1	8	0	0
Epinephrine in oil, subcut.	40	1	5	0	0
Arterenol subcutaneous	100	2	10	90	2.5
" "	200	1	5	60	2.5
" "	400	1	5	80	2.5
" "	500	1	5	80	2.5
" "	1000	1	5	80	1.0
" "	2500	1	5	20	trace
" "	3000	1	5	0	0

**Discussion.** Gibbs, Hanzlik and Tainter<sup>4,7</sup> found that sympathetic nerve stimulation, drugs which cause release of epinephrine from the adrenal medulla, and vasoconstrictor drugs all inhibit *p*-phenylenediamine edema. Egg-white edema is similar in this respect. Selye's<sup>1</sup> and Leger and Masson's<sup>11</sup> observations of a protective effect of "alarming stimuli" might be interpreted as a result of release of epinephrine from the adrenal medulla. Experiments designed to elucidate this point are in progress.

In the course of this work, it was observed that intravenous Evans' blue dye was extravasated into the edematous areas of the egg-white response, notably the feet, ears, nose, lips and tongue. This is similar to *p*-phenylenediamine injury.<sup>6</sup> In both types of edema, *l*-epinephrine is more inhibitory than other pressor substances studied thus far. The present study would indicate therefore that *l*-epinephrine is a much more powerful arteriolar vasoconstrictor (depressor of capillary blood pressure) than *l*-arterenol. In spite of this, the latter has been reported to be 1 to 5 times more active than *l*-epinephrine in raising the arterial blood pressure<sup>14-16</sup> and has been

thought to be the augmentor hormone of the sympathetic nervous system.

The antihistamines tested, pyribenzamine and phenergan, were 1/100 and 1/250 as active, respectively, as *l*-epinephrine, in preventing the edema.

**Summary.** *l*-epinephrine in a subcutaneous dose, 100  $\mu\text{g}$  per kg, inhibits eggwhite edema in the rat. *l*-arterenol is about 1/25 as effective. Isuprel is inactive.

Since submitting the present manuscript, several papers have appeared which have demonstrated the presence of considerable quantities of *l*-arterenol in naturally derived epinephrine preparations, such as the one used in the present study. The use of synthetic *l*-epinephrine, therefore, would have enhanced the difference in activity of *l*-epinephrine and *l*-arterenol reported here.

<sup>14</sup> Tainter, M. L., Tuller, B. F., and Luduena, F. P., *Science*, 1948, **107**, 39.

<sup>15</sup> Euler, U. S. von, *Acta Physiol. Scand.*, 1948, **16**, 63.

<sup>16</sup> Luduena, F. P., Ananenko, E., Siegmund, O. H., and Miller, L. C., *J. Pharm. Exp. Therap.*, in press.

# 17090. Effect of *l*-Epinephrine and *l*-Arterenol on Egg White Edema in the Rat.\*

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A number of reports<sup>1,2,11</sup> have described an extensive edema resulting from the intraperitoneal or intravenous administration of egg white in the rat. The cause of the edema is unknown. There is no evidence that it results from a foreign protein reaction as part of a protein hypersensitivity peculiar to the species. The edema may be of a type similar to *p*-phenylenediamine edema which has a peculiar cephalic distribution in cats, rabbits<sup>3-9</sup> and man<sup>10</sup> and which does not depend on previous exposure to the causative agent.

Selye<sup>1</sup> and Leger and Masson<sup>11</sup> observed that "alarming stimuli" inhibit the reaction and the latter workers also described a protective effect of antihistamines in large doses as did Brown and Werner<sup>12</sup> a fact which we have confirmed.<sup>†</sup> We have noticed that the edema is prevented by much smaller amounts of phenylethylamine pressor drugs and the

object of the present communication is to record this observation.

**Experimental.** Freshly filtered or lyophilized and redissolved raw eggwhite in doses of 0.5 ml was injected intraperitoneally into albino rats of the Slonaker strain, of either sex, and of body weight ranges of 100 to 400 g. The incidence and degree of the edema of the face and extremities were estimated three hours afterward. The incidence was expressed as percentage and the degree by the 0 to 4+ method, averaging the reactions of the face, forefeet and hind feet.

The drugs were injected subcutaneously at the same time as the eggwhite. The drugs reported here included *l*-epinephrine-HCl (Parke, Davis) and *l*-arterenol-HCl (Sterling-Winthrop).

In supplementary experiments to determine the effect of injection route and solvents, *l*-epinephrine-HCl in saline was injected intramuscularly 30 minutes after the eggwhite, and in another experiment subcutaneously in oil.<sup>13</sup> In both cases a comparison was made with the effect of epinephrine injected subcutaneously at the same time as the eggwhite.

**Results.** The results are summarized in Table I which includes 403 rats.

The results show that under the experimental conditions used, the minimal preventative dose of *l*-epinephrine-HCl is about 100 micrograms/kg when injected subcutaneously at the same time as the eggwhite. When injected intramuscularly, 30 minutes after eggwhite, a slightly greater protection occurred; and when injected subcutaneously in oil, the protective dose was about half this. The minimal preventative dose of *l*-arterenol-HCl was about 2500 µg/kg, thus, this substance is about 1/25 as active as *l*-epinephrine-HCl. Isuprel-HCl [*l*-(3',4'-dihydroxyphenyl)-2-isopropylaminoethanol-HCl], a "sympathin-I-mimetic" depressor amine, was inactive up to doses of 25 mg/kg which were toxic.

\* Supported by a grant from the Life Insurance Medical Research Fund.

<sup>1</sup> Selye, H., *Endocrinol.*, 1937, **21**, 169.

<sup>2</sup> Leger, J., Masson, G., and Prado, J. L., *Proc. Soc. Exp. Biol. and Med.*, 1947, **64**, 366.

<sup>3</sup> Hanzlik, P. J., *J. Ind. Hyg.*, 1923, **4**, 386.

<sup>4</sup> Gibbs, O. S., *J. Pharm. Exp. Therap.*, 1923, **20**, 221.

<sup>5</sup> Tainter, M. L., and Hanzlik, P. J., *ibid.*, 1924, **24**, 179.

<sup>6</sup> Tainter, M. L., *ibid.*, 1926, **27**, 201.

<sup>7</sup> Tainter, M. L., *ibid.*, 1928, **33**, 129.

<sup>8</sup> Gibbs, O. S., *ibid.*, 1931, **42**, 65.

<sup>9</sup> Cohen, M. B., *ibid.*, 1933, **48**, 235.

<sup>10</sup> Malosetti, H., Fernandez, G. J., and Migliaro, E., *Arch. Uruguayos Med. Cir. y Espec.*, 1947, **31**, 25.

<sup>11</sup> Leger, J., and Masson, G., *Ann. Allergy*, 1948, **6**, 131.

<sup>12</sup> Brown, B. B., and Werner, H. W., *J. Lab. Clin. Med.*, 1948, **33**, 325.

<sup>†</sup> Pyribenzamine and phenergan [(dimethyl-amino-2-methyl-1-ethyl)-*N*-dibenzo-*p*-thiazine] were tested. The latter was over twice as effective as the former. 10 mg/kg administered subcutaneously at the same time as the eggwhite injection nearly completely inhibiting the edema, while for pyribenzamine, 25 mg/kg were required.

<sup>13</sup> Keeney, E. L., *Bull. Johns Hopkins Hosp.*, 1938, **62**, 227.

TABLE I.  
 $R_f$  Values.

Compounds	Phenol		Collidin	
	65%	80%	71%	63%
DL-valine	0.73	0.69	0.25	0.43
Ethyl-N-DL-valine	0.94		0.41	
Isopropyl-N-DL-valine	0.97		0.43	
Propyl-N-DL-valine	0.99		0.44	
Phenyl-N-DL-valine*				
DL-leucine	0.81	0.74	0.37	0.55
Ethyl-N-DL-leucine	0.92		0.46	
Isopropyl-N-DL-leucine	0.96		0.56	
Propyl-N-DL-leucine	0.99		0.58	
Phenyl-N-DL-leucine*				
DL-phenylalanine	0.83	0.76	0.39	0.58
Ethyl-N-DL-phenylalanine	0.94		0.53	
Isopropyl-N-DL-phenylalanine	0.97		0.59	
Propyl-N-DL-phenylalanine	0.99		0.61	
Phenyl-N-DL-phenylalanine*				

\* The phenyl derivatives evaporated from the paper upon drying (see text).

collidine and with phenol afterwards. It is important, as other authors have also suggested, that one run a control chromatogram of a known amino acid, to establish the necessary correction for the  $R_f$  values of the solvent mixtures used.

Of further interest is the observed degree of ninhydrin color sensitivity of the N-substituted amino acids. Recently Dent<sup>3</sup> reported that sarcosine reacts with an intense color formation. Plattner and Nager<sup>4</sup> have observed positive ninhydrin reactions with methyl-N-DL-valine and methyl-N-L-leucine. It was found that these compounds boiled with ninhydrin did not produce any aldehydes or ketones, because they do not have two hydrogen atoms on the nitrogen. The appearance of

the ninhydrin reaction was positive irrespective of whether the paper was exposed to ultraviolet light or not. The positive ninhydrin color may be interpreted as being the result of a certain degree of hydrolysis that takes place under the effects of the solvents and heating that are used to develop the chromatograms. The intensity of the reaction decreases with increase of chain length in the homologous series of alkyl substitutions. The  $R_f$  values, on the other hand, increase with the length of the substituents, in as much as the n-alkyl group exhibits a somewhat faster rate of movement than the corresponding branched chain isomer.

**Summary.** N-substituted amino acid can be separated by paper chromatographic method. Also, N-substitution leads to the extinction of fluorescence of amino acids.

Received April 20, 1949. P.S.E.B.M., 1949, 71.

<sup>3</sup> Dent, C. E., *Biochem. J.*, 1948, 43, 169.

<sup>4</sup> Plattner, P. A., and Nager, U., *Helv. Chim. Acta*, 1948, 31, 2203.

# 17091. Paper Chromatographic Identification of Some N-substituted Amino Acids.\*

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Ever since Consden, Gordon and Martin<sup>1</sup> introduced the use of paper chromatography it has been successfully applied to the resolution of a great many biological mixtures. In the work presented here, paper chromatography was applied to the separation of a group of amino acid analogs studied for their tumor growth inhibitory effect on the transplanted sarcoma 37 in strain "A" mice.

The ascending capillary technic of Williams and Kirby<sup>2</sup> was adopted for the study of the separation of N-substituted amino acids in quantities of 25  $\gamma$ . The paper strips were suspended from paper clips carried by a toothed non-corroding nickel alloy support which was firmly attached to the heavy glass of the jar. This toothed support permitted the simultaneous employment of 6 strips of Whatman No. 1 filter paper (40  $\times$  12 cm). The solvent mixtures employed were phenol-water and collidine-water. After the solvents reached a 20 cm liquid front (L.F.) value which was within 20 hours, the strips were removed and dried in air with the aid of an electric fan. In developing the ninhydrin reaction the papers were kept at 90° for 10 minutes. The dried strips were then examined under an ultraviolet lamp (General Electric bulb BH4). The free amino acids showed up as faint blue spots, whereas the N-substituted amino acids were recognized by the presence of absorbing, non-emitting dark spots on the paper. After the ultraviolet analysis, the paper strips were sprayed with a 0.15% ninhydrin solution in saturated butanol-water.

Both the free amino acids and substituted analogs responded with spot formation on the

positions outlined by previous ultraviolet analysis. The N-substituted analogs, however, gave spots far below the intensity of those of their corresponding amino acids. It was found that both color intensity and  $R_f$  values could be correlated with the rate of hydrolysis of the substituent and with the character of the steric arrangement. Thus, the size of the isopropyl chain is somewhat smaller than that of the propyl, which expresses itself in the somewhat greater  $R_f$  value of the latter. The phenyl N-substituted amino acids, however, disappeared from the paper either by this method of drying or by heat drying. The phenyl derivatives showed definite melting points between 110 and 180° and were quite soluble in organic solvents. Thus, it is very likely that the small amounts used are removed along with the solvent vapors from the paper strips during drying before any spot development can be observed, for, if control paper strips, untreated with solvent mixtures, carrying the phenyl derivatives were treated with ninhydrin solution, spots characteristic of traces of unsubstituted amino acid showed up, just as in the case of the other N-substituted amino acid-analogs.

Table I represents the tabulated  $R_f$  values of certain of the N-substituted and of the corresponding free amino acids. It must be emphasized that the spots developed by ninhydrin can not be attributed to the presence of traces of amino acids as impurities, as synthesis of the analogs did not employ amino acids at any stage of the work. Furthermore, the microanalytical values of the compounds agreed closely with the calculated ones. As the table shows,  $R_f$  values vary greatly according to the concentration of water in the solvent mixtures. In 65% phenol the N-substituted amino acids move almost as fast as the liquid front. Separation is not sharp and use of this solvent mixture becomes practical only in two dimensional work provided the chromatogram is developed first with 71%

\* This work was supported by a grant from the Cancer Research Grants Branch, U. S. Public Health Service, to Dr. David M. Greenberg.

† U. S. Public Health Special Fellow.

<sup>1</sup> Consden, R., Gordon, A. H., and Martin, A. J. P., *Biochem. J.*, 1944, **38**, 224.

<sup>2</sup> Williams, R. J., and Kirby, H., *Science*, 1948, **107**, 481.

TABLE I.

Effect of Different Amounts of Potassium Acetate and Magnesium Oxide on Growth of Guinea Pigs Receiving a Purified Basal Ration Plus 15% Gum Arabic.

Potassium acetate, %	Magnesium oxide, %	No. of animals	Avg gain in wt during 6 wks, g/day
None	None	4	5.2
2.5	0.25	4	5.3
2.5	0.5	5	7.2
2.5	1.0	4	5.1
5.0	1.0	4	6.2
1.25	0.5	3	6.2

TABLE II.

Growth of Guinea Pigs on Different Rations.

Ration	No. of animals	Avg gain in wt during 6 wks, g/day
Basal + gum arabic	4	5.2
Basal + potassium acetate + magnesium oxide	4	2.6
Basal + gum arabic + potassium acetate + magnesium oxide	13	6.4
Basal + gum arabic + potassium acetate + magnesium carbonate	4	6.2
Basal + gum arabic + potassium acetate + magnesium acetate	4	7.1
Basal + gum arabic + potassium acetate + magnesium sulfate	4	5.4
Basal + gum arabic + potassium bicarbonate + magnesium oxide	4	5.2
Basal + gum arabic + potassium dihydrogen phosphate + magnesium oxide	3	5.5
Rockland stock diet	9	6.6

ment lasting 11 weeks showed that the "normal" growth on the ration containing potassium acetate and magnesium oxide continued at least this length of time. Of the other salts tested magnesium acetate showed a very good growth response. Further experiments with larger numbers of animals are required, however, to show the comparative values of different salts.

Since potassium acetate and magnesium oxide were not active in the absence of the gum arabic, it appears that they may produce conditions in the tract which allow the presence of an intestinal flora more beneficial to the host. The assumption that they may

raise the pH in the cecum is probably not correct because it was found that the pH of the cecum was the same (average 6.9) irrespective of whether or not the diet contained added K and Mg.

*Summary.* A normal rate of growth for guinea pigs, comparable with the growth on commercial rations, was obtained when potassium acetate and magnesium oxide were added to a purified basal ration containing all of the known nutrients (except vitamin B<sub>12</sub>) and 15% gum arabic. The best results were obtained with 2.5% potassium acetate and 0.5% magnesium oxide.

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## 17092. Importance of Potassium and Magnesium in Nutrition of the Guinea Pig.\*

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Several extensive studies have been made to devise a purified ration which will give growth in guinea pigs equivalent to that obtained with commercial rations (about 7 g/day). Booth *et al.*<sup>1</sup> recently found that powdered gum arabic, when added to a purified synthetic ration at a level of 15%, increased the daily growth from 1.8 to 5.1 g. When in addition to gum arabic the ash from alfalfa leaf meal was added to the ration at a level equivalent to 25% of alfalfa, nearly "normal" growth was obtained. The particular constituents of alfalfa ash responsible for the activity observed were not identified. In this paper we report experiments which were conducted to determine the active components of alfalfa ash.

**Experimental.** The experimental procedure was similar to that of Booth *et al.*<sup>1</sup> The synthetic basal ration consisted of: sucrose 60.9%, casein (vitamin-free) 30%, salts IV<sup>2</sup> 4%, fortified soybean oil 4%, sucrose mixture containing B vitamins 0.8%, and choline 0.3%. The fortified soybean oil supplied 1.2 mg of  $\beta$ -carotene, 12 mg of  $\alpha$ -tocopherol, 8  $\mu$ g of calciferol and 0.2 mg of menadione per 100 grams of ration. The vitamin B mixture supplied 1 mg of thiamine hydrochloride, 1.4 mg riboflavin, 1 mg pyridoxine, 3 mg calcium pantothenate, 200 mg inositol, 10 mg niacin, 10 mg p-aminobenzoic acid, 0.04 mg biotin

and 0.3 mg folic acid per 100 grams of ration. Vitamin C dissolved in sucrose solution was fed with individual pipettes at a level of about 25 mg every other day. Gum arabic was used at a level of 15%. The gum and the different inorganic compounds tested were added to the ration in place of an equal amount of sucrose.

**Results.** Since it seemed possible that the beneficial effect of alfalfa ash observed by Booth *et al.* was due to its alkalinity, certain alkaline compounds were used in place of the ash. It was noticed in preliminary experiments that the addition of potassium acetate or magnesium oxide to the basal ration containing gum arabic distinctly improved the growth. Very good growth was obtained with mixtures of these two minerals. The results of an experiment in which different levels of potassium acetate and magnesium oxide were fed are given in Table I. The best growth was obtained when 2.5% potassium acetate and 0.5% magnesium oxide, *i.e.*, 1.0% K and 0.3% Mg, were added to the ration. The amounts of K and Mg in the basal ration supplied by salts IV are 0.56% and 0.04%, respectively.

Table II shows the results of experiments in which other compounds of K and Mg were compared with potassium acetate and magnesium oxide. The amount of potassium acetate used in the rations given in Table II was 2.5% and an equivalent amount of potassium was given as potassium bicarbonate and phosphate. Magnesium oxide was used at a level of 0.5% and an equivalent amount of magnesium was included in the other magnesium compounds. The data show that the growth of the animals receiving potassium acetate and magnesium oxide but no gum arabic was rather poor. However, growth equal to that of animals on the stock ration was obtained, when these minerals were fed in the presence of gum arabic. An experi-

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<sup>†</sup> Rockefeller Foundation Fellow.

<sup>1</sup> Booth, A. N., Elvehjem, C. A., and Hart, E. B., *J. Nutrition*, 1949, **37**, 263.

<sup>2</sup> Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1941, **138**, 459.



FIG. 1a. A medium trophozoite of *P. lophurae* in a mature mouse erythrocyte.  $\times 1770$ .

FIG. 1b. A segmenting parasite of *P. lophurae* in a slightly enlarged mouse erythrocyte, from a smear made 36 hours following introduction of mammalian cells.  $\times 1600$ .

which times avian cell infections averaged about 8000 per 10,000 red blood cells. Five to 11 merozoites, smaller than those in avian erythrocytes, were produced, generally in a rosette form. No gametocytes were found.

Cultures prepared according to the formula of Trager<sup>1</sup> using whole mouse blood in conjunction with infected chick embryo blood indicated a certain amount of invasion of mouse erythrocytes *in vitro*.

In view of the high degree of host specificity of avian malarias, it is surprising that cells

of hosts so distantly related as to be placed in another phylum are invaded. Parasitism of mouse cells might, therefore, demonstrate that in certain cases the resistant principle lies in the serum constituents, rather than in the erythrocyte. *P. lophurae* is transmitted readily by *Anopheles quadrimaculatus* and this fact, along with the successful infection of mouse erythrocytes, might indicate a closer relationship between *P. lophurae* and mammalian malaria parasites than was previously supposed.

<sup>1</sup> Trager, W., *J. Parasitol.*, 1947, **33**, 345.

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#### 17094. A Falling Sphere Method for Studying Clotting in Systems of Purified Blood Components.

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(Introduced by Francis O. Schmitt.)

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Although the conversion by thrombin of fibrinogen to fibrin is generally used as an indicator of reactions occurring earlier in the process of blood coagulation, methods for the study of this conversion, with very few exceptions, measure only a single end-point in fibrin formation.<sup>1</sup> It is apparent that a method which measures quantitative changes

in the coagulation system up to some definite end-point might be of value in following the kinetics of the system. An involved technic has been reported<sup>2</sup> for measuring early alterations in clotting systems by recording changes in intensity of transmitted light as coagulation proceeds. Methods based upon changes in viscosity of a clotting solution have

<sup>1</sup> Quick, A. J., *The Hemorrhagic Diseases and the Physiology of Hemostasis*, Charles C. Thomas, Springfield, 1942.

<sup>2</sup> Nygaard, K. K., *Hemorrhagic Disease*. Photoelectric Study of Blood Coagulation, C. V. Mosby Company, St. Louis, 1941.

# 17093 P. Infection of Mammalian Erythrocytes by the Avian Malaria Parasite, *Plasmodium lophurae*.\*

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Reports concerning attempted infection of mammals with avian malaria parasites are limited, although recently Beckman obtained negative results following introduction of *Plasmodium cathemerium* parasites into man<sup>1</sup> and the guinea pig.<sup>2</sup> In a series of experiments unrelated to the immediate problem, the author noted that guinea pig erythrocytes injected into normal 10-day chick embryos survived for a period of 4 days. As a result of this finding, it was decided to investigate the possibility of securing infection of mammalian cells in chick embryos infected with *P. lophurae*, a parasite to which chick embryos have been found to be highly susceptible.<sup>3</sup>

White leghorn embryos of 10 days' incubation were given intravenous injections of  $3 \times 10^7$  parasites, representing the 24th passage of *P. lophurae* in embryos. Two tenths cc each of twice washed erythrocytes of mouse, rat, rabbit, dog, sheep, and man were injected intravenously into embryos on the 2nd day after parasite introduction, at which time the infections averaged about 1100 per 10,000 avian erythrocytes. Blood films were usually made twice daily thereafter, although in certain embryos 4 or 5 smears were made in a 24 hour period. Giemsa's stain was used. Only cells agreeing in size, morphology, and staining reactions with the mammalian cell introduced were considered in determination of infection, since during the later stages of embryo infection artifacts arose which might have led to false identification.

Dog and rabbit erythrocytes were not well adapted to survival in chick embryos, the

former killing the embryo in a period of 12 hours, the latter disintegrating rapidly. Sheep and rat red blood cells were well preserved even after 3 days but were uninfected. Guinea pig cells, although surviving better than those of the rabbit, disintegrated to the point that they were difficult to separate from abnormal avian erythrocytes. Therefore, although many infected cells suggesting those of the guinea pig were seen, the results were considered negative. Human erythrocytes retained their characteristic morphology throughout the period studied. Two infected cells were found, both with early trophozoites of *P. lophurae*. Infections were so low, however, that this type of cell was regarded as unsuitable for further study.

Erythrocytes of Swiss mice maintained their morphology and were readily parasitized. The proportion of mammalian to avian erythrocytes was usually 1:2. Infected mouse cells were found as early as 12 hours after introduction, although the percentage of infected erythrocytes remained low never exceeding 10 per 10,000. Very few doubly infected cells were observed. Early trophozoites were similar to corresponding stages in the avian erythrocytes. More mature trophozoites frequently elongated into the band forms characteristic of *P. malariae* but, like this parasite, they were sometimes seen as spherical masses in the center of the host cell (Fig. 1a). Pigment granules were small, golden-brown spheres. The cytoplasm was homogenous and stained similarly to *P. lophurae* parasites in chicken erythrocytes. Nuclear structures of trophozoites were identical with *P. lophurae* in chicken red blood cells. After two nuclear divisions parasites occupied a large proportion of the host cell and in later stages caused the cell to enlarge. Pre-segmenting forms with 7-10 nuclei were seen after 32 hours, and fully segmented parasites were seen 36 hours following introduction of erythrocytes (Fig. 1b) at

\* The author wishes to express his gratitude to Dr. W. Trager for constructive criticism and generous assistance throughout this work.

<sup>1</sup> Beckman, H., Proc. Soc. Exp. Biol. and Med., 1947, 66, 401.

<sup>2</sup> Beckman, H., Proc. Soc. Exp. Biol. and Med., 1948, 67, 172.

<sup>3</sup> McGhee, R. B., J. Parasitol., 1949, in press.

tion became opaque a plexiglass bead\* (moistened with saline) was inserted in the upper end of the tube and allowed to fall to an abrupt stop. The elapsed time from mixing of solutions to this sharp end-point was taken as the clotting time. Fibrin or air bubbles clinging to the bead disturbed its fall and rendered the end-point uncertain. The pH was 6.2 in both clotted and unclotted preparations.

*Results. Series A* (Fig. 1) shows the statistical variation in 151 runs before any standardization of the technic as described above was made. The arithmetical mean is 56.2 seconds and the mode is the same indicating a normal distribution curve. The standard deviation is 2.87%.

*Series B* shows the scatter of experimental data obtained in 113 runs after standardization of the technic but employing fibrinogen solutions varying in age from only a few minutes to several hours. The arithmetical mean is 55.7 seconds, the mode is 55.2 seconds and the standard deviation is 2.5%.

*Series C* includes data of 29 runs in which the variation caused by deterioration of the fibrinogen solution was reduced by using a freshly prepared solution every 10 runs (the solution was always less than one and a half hours old when used). The arithmetical mean is 53.5 seconds, the mode is 55.3 seconds and the standard deviation is only 0.98%.

*Series D* shows the accuracy obtained when the errors of weighing, diluting, etc. of different samples are minimized by using the same solutions of fibrinogen and thrombin throughout 12 runs. The runs were made in less than one and a half hours in order to minimize the effect of aging on the thrombin and the fibrinogen. The arithmetical mean is 54.1 seconds, the mode is 54.1 seconds and the standard deviation is 0.49%.

Deterioration of fibrinogen and thrombin in solutions standing at 25°C was manifested by a gradual prolongation of the clotting time. Fig. 2 illustrates the rapid increase in clotting

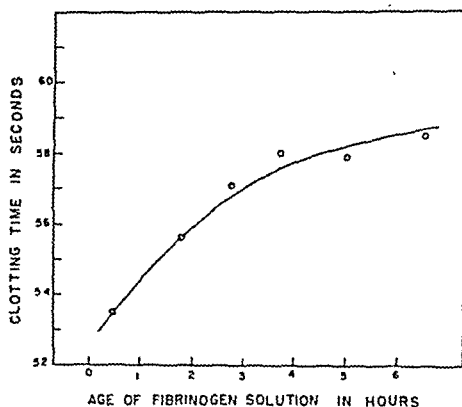


FIG. 2.

The clotting time is shown to increase with the age of a fibrinogen solution allowed to stand over 6 hours at 25°C.

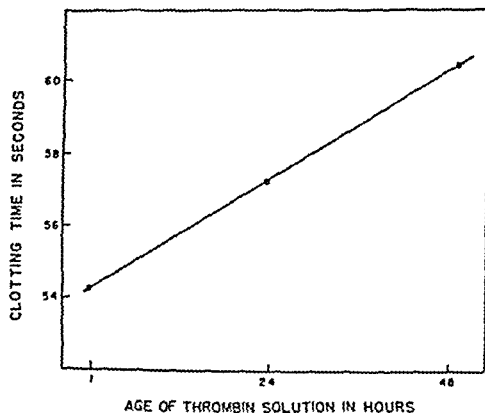


FIG. 3.

Clotting time is shown to increase with the age of a thrombin solution allowed to stand over 48 hours at 4°C.

time of a fibrinogen solution as demonstrated by coagulating aliquots of it at successive intervals over a period of six hours using a fresh thrombin solution. The activity of thrombin solutions also changed on standing, but at a very low rate as illustrated in Fig. 3.

By inserting successive beads at various intervals before gelation was complete and timing the rate of fall of the beads over a measured length of tube, the data for the kinetics curve of Fig. 4 were obtained. The rate of fall of the bead is plotted as a function of the duration of the action of the thrombin upon the fibrinogen. There was no apparent change in the viscosity of the mixture

\* Spherical beads were made of plexiglass (Lucite),  $3.25 \pm 0.1$  mm in diameter and weighing 21.6 mg. They were standardized for uniformity by timing their fall over a definite distance in a tube filled with fibrinogen solution.

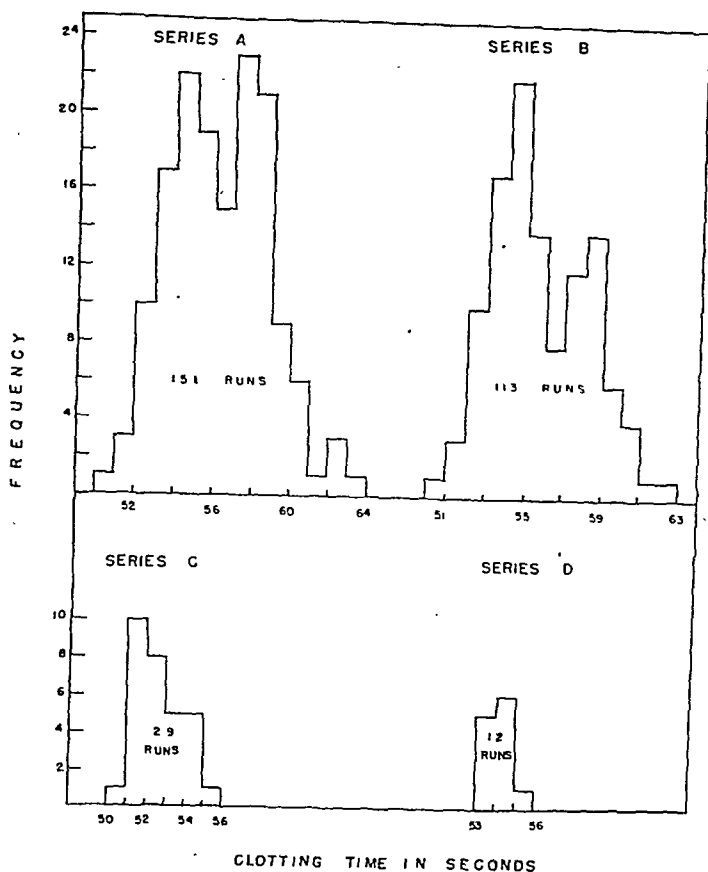


FIG. 1.

Frequency distribution curves of coagulation times, as measured by the falling sphere method, of many different experiments on the thrombin-fibrinogen system. For the experimental conditions of each series of runs, see text.

been described but not in quantitative terms.<sup>3</sup> The purpose of this study was to develop and standardize a quantitative technic for measuring coagulation employing purified thrombin and fibrinogen. This method is based upon the measurement of gelation which occurs in a system during the conversion of fibrinogen to fibrin and utilizes Stoke's law for a falling sphere in the measurement of viscosity.

**Materials and methods.** Bovine thrombin (Upjohn) was dissolved in 0.85% NaCl solution in a concentration of 20 units per milliliter immediately before use. Fraction I of bovine plasma (Armour) was employed as

fibrinogen in a concentration of 0.2% in 0.85% NaCl solution; the fresh solution was filtered through analytical filter paper before using. After adjusting all preparations to  $25 \pm 0.3^\circ\text{C}$  by immersing in a thermostatically controlled water bath, three-tenths milliliter of fresh thrombin solution was added to fifteen milliliters of fibrinogen solution at zero time and rapidly mixed by a rotary movement of the beaker. A pyrex glass tube eight millimeters in diameter, forty centimeters long and stoppered at one end was filled to the brim with the mixture and placed vertically in the water bath with about 3 cm of the upper end of the tube above the surface. As the solu-

<sup>3</sup> Hedenius, P., *Acta med. Scand.*, 1936, **88**, 440.

glutinins which might be present in the serum. (2) After the first stage of the reaction was completed and before the precipitin serum was added, the sensitized cells were washed several times with large volumes of saline in order to remove the supernatant serum proteins which could inhibit the reaction. Moreschi thought that his test served to accentuate the reaction of immune *agglutinins*. Actually, we now know, principally as result of the recent work on Rh antisera, that the test is specific for so-called *univalent antibodies* or *glutinins*.

Following the demonstration by one of us<sup>2,3</sup> that Rh-negative individuals sensitized to the Rh factor may produce two sorts of Rh antibodies, namely, Rh agglutinins (bivalent antibodies) and Rh blocking antibodies or agglutinins (univalent antibodies), Coombs *et al.*<sup>4</sup> suggested the application of the anti-globulin test as a means of detecting univalent Rh antibodies. The purpose of the present communication is to describe a modification of the anti-globulin test by which it is possible to identify human serum globulin specifically and estimate its concentration.

**Materials and method.** The principle of the absorbed, diluted anti-human-globulin is as follows. The solution to be tested for the presence of serum globulin is mixed with the absorbed, diluted anti-human-globulin serum and the mixture allowed to react in a water bath at 37°C for one hour. Then a suspension of Rh-positive cells, coated with Rh<sub>0</sub> univalent antibody, is added and the mixture allowed to stand for a second period of incubation, after which the reaction is read. Failure of clumping to occur indicates the presence of human serum globulin in the material being tested; the occurrence of clumping indicates the absence of human serum globulin.

The sensitized Rh-positive cells used in the test were prepared as follows. A fresh, 2% suspension of Rh<sub>0</sub>-positive red cells in saline was prepared and washed once with saline solution. This suspension was mixed with an

equal volume of an Rh<sub>0</sub> antiserum containing univalent antibodies with a titer of approximately 100 units by the albumin-plasma method.<sup>5</sup> This mixture was allowed to react in the incubator at 37°C for 45 minutes. The tube was then centrifuged at moderate speed, the supernatant fluid discarded, and the sedimented cells washed four times with large volumes of saline solution. The sensitized cells were then resuspended in sufficient saline to make a concentration of approximately 2%.

The antiglobulin serum used in these experiments had been prepared in the usual way and was kindly provided by Drs. Peter Vogel and Richard Rosenfield.

In the experiments to be described the following materials were tested: normal human adult and umbilical cord serum; human spinal fluid, saliva, semen and urine; and normal animal serum from horse, ox, rabbit, and rhesus monkey. The tests were carried out quantitatively by preparing progressively doubled dilutions in saline solution of the material to be tested and mixing a drop of each dilution, in a corresponding series of small test tubes, with a drop of the anti-human globulin serum. After 45 minutes incubation at 37°C, a drop of the suspension of Rh-positive, sensitized cells was added to each tube; the tubes were shaken and reincubated for 60 to 90 minutes. The tubes are then gently shaken and the reactions read with the naked eye and under the low power of the microscope.

**Results.** Table I contains a selection of the results obtained in the various experiments. The tests shown in the table were not all done on the same day but are combined for purpose of comparison.

With the technic used, normal adult human serum completely inhibits the agglutination reaction of the anti-human-globulin serum in dilutions as high as 1:500, on the average. It is necessary to emphasize at the onset that the method shares the limitations of all other serological titrations, namely, a lack of precise reproducibility. In tests made on different days or even on the same day, variations in titer as great as two serum dilutions

<sup>2</sup> Wiener, A. S., *Proc. Soc. Exp. Biol. and Med.*, 1944, **50**, 173.

<sup>3</sup> Wiener, A. S., *J. Lab. and Clin. Med.*, 1945, **30**, 662.

<sup>4</sup> Coombs, R. R. A., Mourant, A. E., and Race, R. R., *Brit. J. Exp. Path.*, 1945, **26**, 255.

<sup>5</sup> Wiener, A. S., and Hurst, J. G., *Exp. Med. and Surg.*, 1947, **5**, 285.

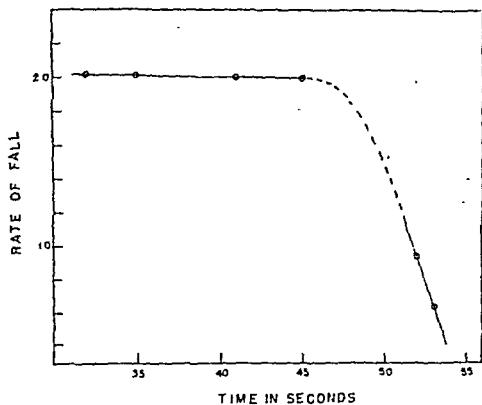


FIG. 4.

The rate of fall of plexiglass beads, expressed in centimeters per second, is plotted as a function of the time elapsing between mixing fibrinogen with thrombin and inserting a bead into the solution. Beads were allowed to fall into the solution 30 seconds after mixing the thrombin and fibrinogen and at 5-10 second intervals after that until gelation had occurred.

for approximately the first 46 seconds of the reaction. Then followed a rapid increase in viscosity leading to gelation and a cessation of falling of the bead. The reproducible clotting times that we have been able to obtain in the statistical study find a ready explanation in the abrupt change in the curve associated with coagulation.

Using 0.3, 0.4, 0.5 and 0.6 ml of thrombin solution (concentration 10 units per ml) average clotting times of 106, 84, 69, and 60

seconds respectively were obtained. (At each dilution the range of readings was  $\pm 3\%$  of the average clotting time). These results indicate a roughly linear relationship between thrombin concentration and rate of clotting.

Three different commercial lots of fibrinogen gave different absolute clotting times under standard conditions owing partially at least to variation in solubility and "percent of clottable protein" in the plasma fraction I. However, different samples of the same lot gave consistent results. The thrombin showed much less variation of activity between lots.

**Summary.** 1. To study the coagulation of the fibrinogen-thrombin system, a simple rapid method has been developed which measures the time required for the developing coagulum to stop the fall of a plastic bead in a glass tube containing the fibrinogen-thrombin mixture.

2. The "clotting time" thus obtained, increases somewhat with the age of the thrombin and fibrinogen solutions.

3. Kinetic studies of the coagulation process indicate that there is no change in viscosity for a considerable time after the mixing of thrombin and fibrinogen, but that this phase is followed by an abrupt increase in viscosity signifying coagulation of the system.

4. The rate of clotting varies with thrombin concentration in a roughly linear fashion.

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## 17095. A New Serological Test (Inhibition Test) for Human Serum Globulin.

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Moreschi<sup>1</sup> described a special serological technic by which it is possible to demonstrate the presence in immune sera of antibodies for red cells or bacteria in higher titers than by the classic agglutination technic. His technic consisted in first titrating the antiserum against the test blood or bacterial suspension

in saline media and subsequently adding a precipitin serum against the serum of the animal providing the antiserum for the erythrocytes or bacteria. The following precautions were necessary in order that this test might work: (1) The precipitin serum had to be absorbed with the washed erythrocytes or bacteria used as antigen, in order to remove any natural hemagglutinins or bacterial ag-

<sup>1</sup> Moreschi, C., *Centralbl. f. Bakteriol.*, 1908, 46, 49; *ibid.*, 1908, 46, 456.

blood serum. With abnormal spinal fluid such as those obtained from syphilitic patients, higher inhibition titers were obtained, as to be expected. Saliva gave definite inhibition though only in low titer, in conformity with the known presence in this secretion of small amounts of serum globulin. Only doubtful reactions were obtained with normal human semen and urine.

Experiments were also conducted to determine the ability of animal sera to inhibit the anti-human-globulin serum. As shown in the table, serum from horse, ox, and rabbit had no apparent inhibiting action. Rhesus serum weakened the clumping, even when highly diluted, but did not inhibit the reaction completely. This indicates the presence in rhesus serum of a serum globulin chemically related to, though qualitatively different from the globulins in human serum.

*Summary and conclusions.* 1. A new serological method of demonstrating human serum globulin has been described, based on its ability to inhibit the agglutinating action of

anti-human-globulin serum for Rh-positive human red cells coated with Rh<sub>0</sub> univalent antibody.

2. Using this technic, further evidence was obtained that the Rh antibody coating sensitized Rh-positive cells is a serum globulin.

3. With the new technic it is possible to demonstrate the presence in normal spinal fluid, and in saliva of small amounts of serum globulin, in proportion to the known concentration in these fluids. Normal human semen and urine failed to inhibit the antiglobulin serum.

4. Umbilical cord serum inhibited anti-globulin serum to the same titer as adult serum.

5. Serum from horse, ox and rabbit did not inhibit the anti-human-globulin serum, while rhesus monkey serum merely weakened its reactions.

6. The new technic may find application not only in clinical medicine, but also in forensic work for the examination of blood stains, and in studies on biochemical evolution.

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## 17096. Relationship of Catalase Activity to Virulence in *Pasteurella pestis*.

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Huddleson<sup>1</sup> and Merz<sup>2</sup> observed that virulent *Brucella* organisms showed greater catalase activity than avirulent strains. This investigation was undertaken to determine whether a similar relationship exists for *Pasteurella pestis*. It was felt that such findings might be applied to the development of a rapid, inexpensive, *in vitro* method for virulence titration. Furthermore, such a relationship would indicate a basis for approaching the problem of virulence along the lines sug-

gested by Jawetz and Meyer<sup>3</sup> who state, "It appears probable that the final criterion of virulence will have to rest on a biological and chemical identification of the bacterial enzyme systems which are responsible for the multiplication of the plague bacilli in the animal host."

*Procedure.* Huddleson's<sup>1</sup> method was modified as follows to measure the catalase activity of virulent and avirulent strains of *P. pestis*. Standard bacterial suspensions were prepared from twice-washed, 24 hour, heart infusion broth cultures suspended in phosphate buffer and adjusted to a turbidity of  $100 \pm 5$  on the scale of the Klett-Summerson photoelectric

<sup>1</sup> Huddleson, I. F., Univ. Mich. Agri. Exp. Station, Technical Bull. 182, January, 1942.

<sup>2</sup> Merz, P., *Über die Katalasen der Brucellen*, Inaugural Dissertation for the Degree of Doctor of Veterinary Medicine, University of Zurich, 1938.

<sup>3</sup> Jawetz, E., and Meyer, K. F., *Am. J. Path.*, 1944, 20, 457.



TABLE I.  
Results of Inhibition Tests, Using Rabbit Anti-Human-Globulin Serum and Rh-Positive Erythrocytes Coated with Anti-Rh<sub>0</sub> Glutinin.

Material tested	Dilution of material tested													
	Undil.	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	1:5096	1:10,192
Normal human serum (adult)	—	—	—	—	—	—	—	—	—	—	+	+	+	+
“ “ (umbilical cord)	—	—	—	—	—	—	—	—	—	—	+	+	+	+
10% purified human globulin	—	—	—	—	—	—	—	—	—	—	+	+	+	+
25% “ albumin	+	+	+	+	+	+	—	—	—	—	—	—	—	—
Normal human spinal fluid	—	—	—	+	+	+	+	+	+	+	+	+	+	+
Spinal fluid (4+ Wassermann)	—	—	—	+	+	+	+	+	+	+	+	+	+	+
Normal human urine	+	+	—	—	+	+	+	+	+	+	+	+	+	+
“ “ saliva	—	+	—	+	+	+	+	+	+	+	+	+	+	+
“ “ semen	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Normal horse serum	+	+	+	+	+	+	+	+	+	+	+	+	+	+
“ ox	+	+	+	+	+	+	+	+	+	+	+	+	+	+
“ rabbit	+	+	+	+	+	+	+	+	+	+	+	+	+	+
“ rhesus	+	+	+	+	+	+	+	+	+	+	+	+	+	+

were not unusual, so that only a tolerable degree of accuracy was possible by averaging the results of several titrations. In addition the end points of the inhibition titrations were often difficult to determine due to the irregularity of the degree of clumping in successive tubes in many of the experiments.

In an attempt to determine which serum fraction is responsible for the inhibition of the anti-globulin serum, titrations were carried out with purified human serum albumin and purified human serum globulin. The serum albumin used was a 25 percent salt-poor solution purchased from Cutter Laboratories. The serum globulin was a 10 percent solution of Squibb's Red Cross Gamma Globulin, kindly provided by Dr. P. Vogel. As shown in the table, the serum albumin failed to inhibit the anti-globulin serum, while the serum globulin inhibited the serum strongly, in accordance with expectation. These results therefore provide additional proof that the antibody coating the sensitized red cells consists of serum globulin. It is to be noted that the inhibition titer of the purified human globulin is higher than that of unprocessed serum, in proportion to the concentration of globulin.

A few tests were performed with human umbilical cord serum, which gave titers approximately equal to those of normal adult serum. Even assuming that the fetus *in utero* is incapable of producing serum globulin, this finding was to be expected from the fact that univalent maternal antibodies readily traverse the placenta into the fetal circulation until the titers in the maternal and fetal circulation are equal.<sup>6,7</sup>

The normal human spinal fluid inhibited the anti-globulin serum but only up to the dilutions of approximately 1:2 or 1:4. Since normal serum contains approximately 7 g of protein per 100 cc while normal spinal fluid contains only about 30 mg per 100 cc, the inhibition titer of approximately 3 units obtained for spinal fluid corresponds satisfactorily to the expectation, on the basis of an inhibition titer of approximately 500 units for

<sup>6</sup> Wiener, A. S., and Sonn, E. B., *J. Lab. and Clin. Med.*, 1946, **31**, 1016.

<sup>7</sup> Wiener, A. S., *Ann. Allergy*, 1948, **6**, 293.

colorimeter using a green filter (540 mμ) and a 15 mm cuvet. Five ml of this suspension were pipetted into the substrate solution (25 ml of 0.48 N H<sub>2</sub>O<sub>2</sub> in 0.03 M Sorenson phosphate buffer at pH 6.80) in a 250 ml Erlenmeyer flask shaken at 100 oscillations per minute. At 5, 15, 30 and 60 minute intervals, 5 ml samples were removed to beakers containing 3 ml of 7 N H<sub>2</sub>SO<sub>4</sub> and titrated with 0.1 N KMnO<sub>4</sub>. Blank titration showed that no spontaneous decomposition of H<sub>2</sub>O<sub>2</sub> occurred during the experiment. Enzyme activity is proportional to the quantity of H<sub>2</sub>O<sub>2</sub> decomposed.

The viable bacterial count of the standard suspensions was determined by surface plating, and virulence was titrated by injecting 0.2 ml of appropriate dilutions subcutaneously into 8-14 week old mice, 10 mice being used for each of 3 serial dilutions.

**Results.** The results are presented in Table I. Inspection of Table I reveals that the catalase activity of virulent strains was significantly greater than that of avirulent strains. In the 14 virulent strains studied, the enzyme activity, as expressed in ml of 0.10 N H<sub>2</sub>O<sub>2</sub> decomposed in 60 minutes, ranged from 12.9 to 19.9 ml with a mean value of 16.7 ml. On the other hand, for 11 avirulent strains, values of 2.1 to 8.6 ml with a mean value of 5.1 were found. The difference between the mean values of virulent and avirulent organisms is statistically significant.

Some of the factors involved in the catalase activity of *P. pestis* were investigated and the influence of a number of conditions was studied. It was observed in early experiments that growth on such different media as heart infusion broth (Difco), nutrient agar (Difco), or horse blood agar was without effect on catalase activity. The number of viable organisms was observed to undergo a 5-fold decrease when the incubation period was extended from 24 to 72 hours. Despite this decrease, no differences in catalase activity were exhibited by standard suspensions prepared from cultures after 24, 48, or 72 hours of incubation. The stability of the enzyme was also demonstrated by experiments in which standard sus-

pensions were stored at 5°C for as long as seven days without change in activity. A final substrate concentration of 0.40 N H<sub>2</sub>O<sub>2</sub> was found to be most suitable for our purposes. No significant differences in catalase activity were observed over a temperature range of 24-32°C or with changes in shaking rate from stationary to 103 oscillations per minute.

The kinetics of catalase activity in *P. pestis* under the conditions outlined appear to be similar to other findings<sup>4-7</sup> reported for cell-free preparations of this enzyme and apparently follow the curve for a monomolecular reaction (Table I).

The use of catalase activity as an *in vitro* screening test for virulence is suggested by the differences between virulent and avirulent strains. Experiments are being continued to determine whether this test will distinguish varying degrees of virulence during the *in vitro* attenuation of some of the virulent strains studied.

**Summary.** 1. Optimal conditions for catalase activity in *P. pestis* were determined and the catalase activity of 14 virulent and 11 avirulent strains was measured.

2. The catalase activity of virulent strains was significantly greater than that of the avirulent strains.

3. The possibility of using catalase activity as a screening test for determining virulence *in vitro* has been indicated.

The author wishes to acknowledge the technical assistance of Virginia Keppel. He is also indebted to Dr. K. F. Meyer, Director, The George Williams Hooper Foundation for Medical Research, University of California Medical Center, San Francisco, for making available most of the strains used in these studies.

<sup>4</sup> Morgulis, S., *J. Biol. Chem.*, 1921, **47**, 341.

<sup>5</sup> Waksman, S. A., and Davison, W. C., *Enzymes—Properties, Distribution, Methods and Applications*, Williams and Wilkins Co., 1926.

<sup>6</sup> Sumner, J. B., and Somers, G. F., *Chemistry and Methods of Enzymes*, New York Academic Press, 1947.

<sup>7</sup> Von Fuler, H., *General Chemistry of Enzymes*, John Wiley and Sons, London, 1912.

TABLE I.  
Relationship of Catalase Activity to Virulence in *Pasteurella pestis*.

<i>Pasteurella pestis</i> strains		Catalase determinations		Viable bacteria ( $\times 10^8$ /ml)		Virulence		Catalase activity (ml of $H_2O_2$ )			
No.	Name	Catalase determinations		Viable bacteria ( $\times 10^8$ /ml)		No. of titrations	LD <sub>50</sub>	5	15	30	60.
II-16	139-L	8		2.9		5	90	9.7 $\pm$ 1.0	15.4 $\pm$ 1.2	18.1 $\pm$ 1.8	19.3 $\pm$ 0.1
3	Yreka	8		3.3		5	110	10.3 $\pm$ 3.1	14.5 $\pm$ 2.8	16.6 $\pm$ 3.3	19.3 $\pm$ 0.1
14	F 9650	2		3.3		1	90	8.1	11.3	15.4	17.7
18	0 9817-C	2		2.7		1	50	10.8	13.9	16.7	17.7
5	327	8		2.2		5	20	8.5 $\pm$ 1.0	13.2 $\pm$ 2.1	15.6 $\pm$ 2.3	17.3 $\pm$ 0.7
13	1-72	2		2.3		1	30	9.7	12.6	14.8	17.2
1	Shasta (II-1)	2		3.0		2	230	14.6	14.9	15.4	17.1
17	" 412	2		2.6		1	240	6.5	11.2	14.6	16.7
2	" (II-2)	2		2.2		2	140	13.5	13.6	14.3	16.5
10	Webster	2		6.5		2	1100	12.8	13.4	14.7	16.4
12	de Rosa	2		2.4		2	3300	12.8	13.4	14.2	16.2
19	—	2		3.5		1	70	7.0	9.7	12.1	15.5
15	F 9581	2		1.7		—	—	7.4	10.1	13.7	14.7
11	—	2		2.1		1	2200	6.7	9.5	11.3	13.6
								Mean	16.7 $\pm$ 0.4		
C-11	Seemeding	2		8.4		2	†	3.3	6.4	7.9	8.2
5	TRU	2		20.0		2	†	3.3	7.0	7.7	8.0
7	53 II-1	2		9.3		2	†	2.9	6.0	6.6	7.0
4	K-120	2		23.0		2	†	2.0	4.4	5.1	5.2
K-1	A-1122	2		6.9		2	†	3.4	4.7	4.9	5.0
C-12	14	2		8.0		2	†	2.1	4.0	4.6	4.9
6	Tijvidej	2		8.8		2	†	1.6	3.8	4.4	4.7
3	Java	2		5.6		2	†	1.2	3.1	3.8	3.8
10	EV 76	2		7.6		2	†	1.3	2.8	3.4	3.6
8	A 1122	2		11.0		2	†	1.1	2.7	3.1	3.4
9	Bombay	2		8.5		2	†	0.6	1.8	2.2	2.4
								Mean	5.1 $\pm$ 0.5		

\* LD<sub>50</sub>—No. of organisms killing 50% of 8-14 weeks-old mice in 14 days. Reed and Muench.  
† 2  $\times$  10<sup>4</sup> organisms injected subcutaneously into 8-12-weeks-old mice caused no deaths.  
s Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, 27, 493.

TABLE I.  
Oxygen Consumption Changes with Single Liter Intravenous Infusions of 5% Glucose in Normal Saline, of 6% Gelatin Solution, and of 5% Amigen-Dextrose Solution.

Type infusion	PT.		Oxygen consumption changes					
			Pre-inf.	Infusion period			Post-infusion period	
5% Glucose in N S	W B	at hr	0	2	4		5½	24
		cc/Sq M/min	127	121	117		119	126
		% change	0	-4.8	-7.9		-6.1	-0.4
	J S	at hr	0	2	4		5	
		cc/Sq M/min	123	128	124		116	
		% change	0	+4.0	+0.4		-5.7	
6% Gelatin	W B	at hr	0	2	4	5	7	
		cc/Sq M/min	126	120	123	127	124	
		% change	0	-5.2	-2.2	+0.4	-1.7	
5% Amigen in 5% Dextrose	J G	at hr	0	2	4	6		
		cc/Sq M/min	134	135	148	135		
		% change	0	+0.4	+10.0	+0.4		
		at hr	0	2	6	8	10	12*
	H K	cc/Sq M/min	140	136	156	139	144	163
		% change	0	-2.4	+11.2	-0.4	+3.4	+12.1

\* ½ hour after a meal of 350 calories.

TABLE II.  
Oxygen Consumption Changes with Single Liter Intravenous Infusions of 5% and 10% Fat Emulsions (50 and 100 g of Coconut Oil Emulsified with 6% Gelatin Solution).

Infusion fat emulsion	PT		Oxygen consumption changes							
			Pre-inf.	— Infusion period —				— Post-infusion period —		
5%	H K	at hr	0	2	4	6	8	10	12*	24
		cc/Sq M/min	152	183	186	179	160	140	178	154
		% change	0	+21.1	+23.4	+18.4	+5.8	-7.0	+18.3	+1.8
	J L	at hr	0	2	6	8	10	12	14	24
		cc/Sq M/min	113	137	145	154	145	132	122	116
		% change	0	+21.5	+27.6	+36.5	+27.6	+16.0	+7.7	+2.8
10%	J L	at hr	0	2	4	6	8	10	12	24
		cc/Sq M/min	129	147	152	173	147	130	129	126
		% change	0	+14.1	+18.0	+34.1	+5.8	+0.4	0	-1.9
	J S	at hr	0	2	4	6	8	10	12	24
		cc/Sq M/min	142	163	154	166	170	139	126	132
		% change	0	+14.4	+7.8	+16.8	+19.2	-2.3	-11.7	-7.0

\* ½ hour after a meal of 400 calories.

*Results.* The oxygen consumption changes obtained with the 6 types of intravenous preparations are summarized in Tables I, II and III. The percentage changes in oxygen consumption during the infusion and post-infusion periods were computed for purposes of comparison with the initial or pre-infusion value taken as the zero level.

Maximal changes in oxygen consumption generally occurred after more than eighty per cent of any preparation had been infused or upon completion of the infusion period. With 5% glucose in normal saline and 6% gelatin

infusions, the oxygen uptake values were mainly near the pre-infusion level while the recipients of 5% amigen and dextrose solution showed rises to about 11.0%. The recipients of 5% and 10% fat emulsions showed an increased oxygen consumption to levels ranging from 19.2 to 36.5% above their initial values. Elevations in oxygen consumption levels were usually greatest in recipients of the 10% combined fat emulsion with increases from 42.4 to 49.7%. One subject, FS, who received 2 liters of the 10% combined fat emulsion, was found to have in-

# 17097. Oxygen Consumption Studies with Intravenous Infusions of the Combined Fat Emulsion.\*

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*From the Laboratory of Experimental Surgery, Department of Surgery, New York University College of Medicine.*

In previous reports from this laboratory, the results of experimental and clinical investigations of an intravenous fat emulsion combined with protein and glucose were presented.<sup>1-3</sup> The method of preparation of the combined fat emulsion and its effect upon humans were summarized in detail.<sup>3</sup> The present study is concerned with oxygen consumption associated with the intravenous infusion of the combined fat emulsion. Measured changes in oxygen consumption obtained with a variety of other intravenous preparations were made for purposes of comparison and served as control standards. This aspect of energy metabolism was the first of several methods of investigation with a view of obtaining evidence in favor of metabolic utilization of intravenously infused fat.

*Method of study.* Serial oxygen consumption determinations were conducted in a group of seven surgical patients who received one or more of 6 types of intravenous preparations. The patients, all males ranging in age from 18 to 58 years, were drawn from the surgical service. Because of good emotional stability, they were selected as the most suitable for oxygen uptake studies with the basal metabolism machine. Although these patients were on the surgical service because of a variety of diseases, at the time of testing there were no complicating factors of temperature elevation, active infection, chronic decubitus ulcers

or acute organic metabolic disorders. Three different intravenous fat emulsions were studied; Emulsion 1, was a 5% fat emulsion which contained 50 g of coconut oil emulsified with 6% gelatin and provided 450 calories per liter; Emulsion 2, was a 10 per cent fat emulsion which contained 100 g of coconut oil emulsified with 6% gelatin to provide 900 calories per liter and Emulsion 3, always referred to in this paper as the combined fat emulsion, consisted of an homogenate of 10% coconut oil, 5% amigen, 5% glucose and 2% gelatin per liter with a caloric value of approximately 1300 calories. The comparative controls were (a) 5% glucose in normal saline, 200 calories per liter, (b) 6% gelatin solution, caloric value undetermined<sup>4</sup> and (c) 5% amigen and dextrose solution, 350-400 calories per liter.

Each infusion study was started in the morning with the patient in the post-absorptive state after a fasting period of 14 hours. In every case except one, a single liter of test fluid was administered. The duration of the infusion period ranged from 4 to 6 hours with 3 instances of extension up to 8 hours. Serial oxygen consumption determinations were obtained with the Benedict-Roth apparatus and on 2 occasions by the Tissot method. Each infusion study consisted of the determinations of the oxygen consumption level beginning with the pre-infusion period and measured in interval series during the infusion and post-infusion periods. The temperature, pulse, respirations and blood pressure were obtained throughout the study period at hourly intervals. Urine specimens were obtained at regular intervals for the first 14 hours and also at the end of 24 hours. Urinalyses were made for acetone (Rothera's test) and diacetic acid (Gerhardt's test).

\* Aided by a grant from the Ben Lewis Fund in Experimental Surgery. Clinical Investigation done at Goldwater Memorial Hospital (New York University, Third Surgical Division). Technical assistance, J. Baker.

<sup>1</sup> Shafiroff, B. G. P., and Frank, C., *Science*, 1947, **106**, 474.

<sup>2</sup> Shafiroff, B. G. P., Baron, H. C., and Roth, E., *Proc. Soc. Exp. Biol. and Med.*, 1948, **69**, 387.

<sup>3</sup> Shafiroff, B. G. P., Mulholland, J. H., Roth, E., and Baron, H. C., *Proc. Soc. Exp. Biol. and Med.*, 1949, **70**, 343.

<sup>4</sup> Koop, C. E., Riegel, C., Grigger, A. B., and Barnes, M. T., *Surg. Gyn. Obst.*, 1947, **84**, 1065.

TABLE I.  
Oxygen Consumption Changes with Single Liter Intravenous Infusions of 5% Glucose in Normal Saline, of 6% Gelatin Solution, and of 5% Amigen-Dextrose Solution.

Type infusion	PT.		Oxygen consumption changes					
			Pre-inf.	Infusion period			Post-infusion period	
5% Glucose in N S	W B	at hr	0	2	4		5½	24
		cc/Sq M/min	127	121	117		119	126
		% change	0	-4.8	-7.9		-6.1	-0.4
	J S	at hr	0	2	4		5	
		cc/Sq M/min	123	128	124		116	
		% change	0	+4.0	+0.4		-5.7	
6% Gelatin	W B	at hr	0	2	4	5	7	
		cc/Sq M/min	126	120	123	127	124	
		% change	0	-5.2	-2.2	+0.4	-1.7	
5% Amigen in 5% Dextrose	J G	at hr	0	2	4	6		
		cc/Sq M/min	134	135	148	135		
		% change	0	+0.4	+10.0	+0.4		
		at hr	0	2	6	8	10	12*
	H K	cc/Sq M/min	140	136	156	139	144	163
		% change	0	-2.4	+11.2	-0.4	+3.4	+12.1

\* ½ hour after a meal of 350 calories.

TABLE II.  
Oxygen Consumption Changes with Single Liter Intravenous Infusions of 5% and 10% Fat Emulsions (50 and 100 g of Coconut Oil Emulsified with 6% Gelatin Solution).

Infusion fat emulsion	PT		Oxygen consumption changes							
			Pre-inf.	— Infusion period —				— Post-infusion period —		
5%	H K	at hr	0	2	4	6	8	10	12*	24
		cc/Sq M/min	152	183	186	179	160	140	178	154
		% change	0	+21.1	+23.4	+18.4	+5.8	-7.0	+18.3	+1.8
	J L	at hr	0	2	6	8	10	12	14	24
		cc/Sq M/min	113	137	145	154	145	132	122	116
		% change	0	+21.5	+27.6	+36.5	+27.6	+16.0	+7.7	+2.8
10%	J L	at hr	0	2	4	6	8	10	12	24
		cc/Sq M/min	129	147	152	173	147	130	129	126
		% change	0	+14.1	+18.0	+34.1	+5.8	+0.4	0	-1.9
	J S	at hr	0	2	4	6	8	10	12	24
		cc/Sq M/min	142	163	154	166	170	139	126	132
		% change	0	+14.4	+7.8	+16.8	+19.2	-2.3	-11.7	-7.0

\* ½ hour after a meal of 400 calories.

**Results.** The oxygen consumption changes obtained with the 6 types of intravenous preparations are summarized in Tables I, II and III. The percentage changes in oxygen consumption during the infusion and post-infusion periods were computed for purposes of comparison with the initial or pre-infusion value taken as the zero level.

Maximal changes in oxygen consumption generally occurred after more than eighty per cent of any preparation had been infused or upon completion of the infusion period. With 5% glucose in normal saline and 6% gelatin

infusions, the oxygen uptake values were mainly near the pre-infusion level while the recipients of 5% amigen and dextrose solution showed rises to about 11.0%. The recipients of 5% and 10% fat emulsions showed an increased oxygen consumption to levels ranging from 19.2 to 36.5% above their initial values. Elevations in oxygen consumption levels were usually greatest in recipients of the 10% combined fat emulsion with increases from 42.4 to 49.7%. One subject, FS, who received 2 liters of the 10% combined fat emulsion, was found to have in-

## OXYGEN CONSUMPTION WITH INTRAVENOUS FAT

TABLE III.  
Oxygen Consumption Change with Single Liter Intravenous Infusions of the 10% Combined Fat Emulsion (10% coconut oil, 5% anigen, 5% glucose and 5% gelatin solution).

PT.		Pre-inf.	Infusion period			Post-infusion period		
			2	4	7	7	10	14
W B	at hr cc/Sq M/min.* % change	0 167 0	221 +32.3	243 +45.4	193 +15.8	24 152 -9.1		
H K	at hr cc/Sq M/min.* % change	0 167 0	2 209 +25.8	4 225 +34.8	7 171 +2.7	24 152 -9.1		
J L	at hr cc/Sq M/min. % change	0 118 0	2 169 +43.8	4 175 +49.2	6 162 +37.3	8 152 +28.9	10 142 +20.8	12 135 +14.9
S P	at hr cc/Sq M/min. % change	0 116 0	2 159 +36.8	4 165 +42.1	6 167 +43.3	8 137 +17.6		
S P	at hr cc/Sq M/min. % change	0 122 0	2 140 +20.2	4 155 +25.2	6 176 +42.4	8 172 +30.0	10 134 +8.8	12 133 +7.1
** F S	at hr cc/Sq M/min. % change	0 121 0	1½ 155 +28.3	3½ 174 +43.3	5 198 +63.9	6¾ 192 +57.7	9½ 140 +23.2	12½ 120 -40.6
J S	at hr cc/Sq M/min. % change	0 124 0	2 148 +19.8	4 165 +33.8	6 179 +45.0	8 178 +44.1	10 168 +36.0	12 137 +11.3

\* By Tissot method.

\*\* FS received 2 liters.

creased his oxygen uptake to a maximum of 63.9% above his initial level. Another subject, HK was given oral feedings of approximately 350-400 calories at the 12th hour of each of 2 infusion studies as a result of which further elevations in oxygen uptake were again demonstrable.

Urinalysis for acetone and diacetic acid were positive in the intermediate serial samples in 3 patients who received the 5% or 10% emulsion. Tests for acetone were usually 2 and 3 plus in the 4 and 6 hour urine specimens followed by 1 plus to faint traces at 8 hours with negative results for the 10 hour and subsequent specimens. Tests for diacetic acid indicated faint traces only at the height of acetone spillage. Urine specimens were usually negative for ketone bodies in the 24 hour study period in the recipients of the 10% combined fat emulsion. In the latter group, the 4 hour urine specimen was found occasionally to contain a trace of acetone.

Temperature elevations ranging from 99.6 to 100.4° were noted between the tenth and sixteenth hours in the post-infusion period with the combined fat emulsion in the case of JL and SP. With the latter elevations in body temperature correlative secondary rises in oxygen consumption and moderate increases in pulse rate were found to occur. Analysis of temperature, pulse rate, respiration and blood pressure taken in each study at hourly intervals revealed minor fluctuations during the course of any type of infusion without significant findings associative with the oxygen consumption studies.

*Comments.* Evidence in favor of the metabolic utilization of intravenously infused fat has been reported by many investigators. The respiratory metabolism of human infants in relation to infusions of an homogenized fat emulsion was found to be reflected in increased heat production and alteration in the respiratory quotient. The energy exchange was derived from the combustion of the intravenously injected fat.<sup>5</sup> Other evidence in favor of utilization of intravenously infused fat has been obtained with parenterally administered

C<sub>14</sub> labelled tripalmitin emulsion as a result of which more than 50% of the radioactive isotope was recovered in expired carbon dioxide in a 24 hour study period. Proof was also given of incorporation of the labelled fatty acids into phospholipids.<sup>6</sup> In another experimental study data was presented to show that growing puppies obtained up to 30 per cent of their total energy requirement from daily infusions of a fat emulsion which in combination with a fat free diet provided for positive nitrogen balance and weight and growth gain.<sup>7</sup>

Indications of the metabolic utilization of a 10% combined fat emulsion administered intravenously were obtained by means of studies of oxygen consumption. For proper evaluation of the role of fat contained in the emulsion, 5 and 10% concentrations of the emulsified oil were studied. These studies showed that the increased oxygen consumption was not only correlative with fat as the prime contributing energy factor but that additive concentrations of fat raised significantly the amount of oxygen uptake. The non-fat components of the combined fat emulsion, gelatin, amigen and dextrose, were found to show small increments of change from the initial pre-infusion value. By combination of all the basic constituents with fat into a combined emulsion, the greatest increases in oxygen consumption resulted. Transient ketonuria noted in some of our studies were indicative both of early metabolic utilization and incomplete fat metabolism, the ketogenesis developing during the period of fat plethora.

The Benedict-Roth recording spirometer and the Tissot type apparatus were accepted by us as experimentally adequate for the present investigation. Alterations in tidal air as a possible explanation for increased oxygen consumption has also been eliminated in this study.<sup>8</sup>

<sup>6</sup> Lerner, S. R., Chaikoff, I. L., Entenman, C., and Dauben, W. G., *Science*, 1949, **109**, 13.

<sup>7</sup> Mann, G. V., Geyer, R. P., Watkin, D. M., Smythe, R. L., Dsai-Chwen Dju, Zamelchek, N., and Stare, F. J., *J. Lab. and Clin. Med.*, 1948, **33**, 1503.

<sup>5</sup> Gordon, H. H., and Levine, S. Z., *Amer. J. Dis. Child.*, 1935, **50**, 894.

<sup>8</sup> Peters, G. A., Horton, B. T., and Boothby, W. M., *J. Clin. Invest.*, 1945, **24**, 611.



**Summary.** (1) Sixteen serial oxygen consumption studies were conducted in a group of 7 adult humans who received one or more of 6 types of intravenous preparations.

(2) Maximal increases in oxygen consumption above the initial pre-infusion levels attained their greatest values in recipients of the 10% combined fat emulsion when compared with a variety of intravenous preparations of a lesser caloric or energy value.

(3) With fat as the prime contributing factor to the increased energy value of the 10% combined fat emulsion, it is justifiable to conclude that the intravenously infused fat is utilized for energy production when in part it undergoes oxidative combustions which are ultimately measured in terms of oxygen consumed.

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## 17098. Effect of Dibenamine on Blood Flow and Cardiac Output in the Dog.

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Since N, N-dibenzyl- $\beta$ -chloroethylamine (Dibenamine) was first introduced as an adrenergic blocking agent, many reports have been made on various aspects of its pharmacological properties.<sup>1</sup> Evidence has been presented indicating that Dibenamine produces vasodilation as evidenced by an increase in skin temperature,<sup>2</sup> and in dogs subjected to hemorrhage or trauma those pretreated with Dibenamine showed a slightly higher total blood flow than the controls.<sup>3</sup> It also has been reported that Dibenamine has no significant effect on cardiac output,<sup>1</sup> or increases cardiac output,<sup>4</sup> although no data are given on this point. In this report data are presented on the effects of Dibenamine on blood flow and cardiac output.

**Methods.** With but one exception the dogs were anesthetized with barbital sodium 300 mg/kg, chloralose, 250 mg/kg, was used

in one dog. Blood pressure was obtained from a carotid artery with a Hamilton manometer and cardiac output determined from the pressure pulse by the method of Hamilton and Remington.<sup>5</sup> Blood flow was recorded from either a carotid or a femoral artery by means of a rotometer.<sup>6</sup> The anticoagulant used was chlorazol fast pink, 2 cc of an 8% solution per kg. The dose of Dibenamine, 20 mg/kg, when given intravenously (i.v.), was administered slowly over a 5-minute period. In a majority of the dogs the degree of adrenergic action was tested with epinephrine 30 minutes after the Dibenamine injection. In all cases only a depressor response was elicited.

**Results.** Table I summarizes the data on those dogs in which cardiac output, blood pressure and blood flow were determined; pressure and flow were measured in 16 additional dogs. In all of the 24 dogs but 7 the blood pressure dropped significantly, 3 of these showed a rise in pressure and in the other 4 the changes were less than 10 mm Hg. The drop in pressure occurred about 20 minutes after Dibenamine was given. Little effect was noted on heart rate with a slight

<sup>1</sup> Nickerson, M., and Goodman, L. S., *J. Pharm. Exp. Therap.*, 1947, **80**, 167; *Fed. Proc.*, 1948, **7**, 397.

<sup>2</sup> Hecht, H. H., and Anderson, R. B., *Am. J. Med.*, 1947, **3**, 3; Vleeschhouwer, G. R., *Proc. Soc. Exp. Biol. and Med.*, 1947, **66**, 151.

<sup>3</sup> Remington, J. W., Wheeler, N. C., Boyd, G. H., and Caddell, H. M., *Proc. Soc. Exp. Biol. and Med.*, 1948, **69**, 150.

<sup>4</sup> Wiggers, H. C., Ingraham, R. C., Roemhild, F., and Goldberg, H., *Am. J. Physiol.*, 1948, **153**, 511.

<sup>5</sup> Hamilton, W. F., and Remington, J. W., *Am. J. Physiol.*, 1947, **148**, 14.

<sup>6</sup> Gregg, P. E., Shipley, R. E., Eckstein, R. W., Rotta, A., and Wear, J. T., *Proc. Soc. Exp. Biol. and Med.*, 1942, **49**, 267.

TABLE I.  
Effects of Dibenamine on Blood Pressure, Cardiac Output and Peripheral Blood Flow.

Dog	Blood pressure			Cardiac output, liters/M <sup>2</sup>			Blood flow, cc/min.		
	After Dibenamine			After Dibenamine			After Dibenamine		
	Control	30 min.	60 min.	Control	30 min.	60 min.	Control	30 min.	60 min.
DF 13	207/175	155/105	135/60	2.5	3.0	4.8	82	45	41
3	150/128	93/75	91/25	1.5	1.45	1.7	4	8	4
14	190/135	198/112	202/140	3.4	4.0	3.8	60	116	135
15	295/242	102/57	Died	6.0	4.7	Died	148	16	Died
16	190/157	95/77	95/70	1.4	1.3	1.3	82	32	12
17	185/162	130/115	122/107	1.2	1.0	1.0	39	36	41
18	205/180	155/130	152/110	1.9	0.9	2	55	28	30
19	220/187	150/87	145/77	1.8	3.9	3.4	48	39	36

increase being the most frequent response. The blood flow during the experimental period, dropped in 16, remained the same in 6 and increased in 2 dogs one of which had a blood pressure rise of 12 mm Hg and the other a fall of 10 mm Hg. The flow in the other 2 dogs, whose pressure was increased, remained constant in one and dropped 30% in the other. The cardiac output dropped in one case only (DF 15), there was an increase in 2 (DF 13, DF 18), and the others did not change significantly.

Dibenamine was administered intra-arterially in 2 to 4 mg/kg amounts in 3 dogs. There was no evidence of any peripheral action.

In 2 dogs epinephrine, 0.01 mg/kg was given at the end of the 30 minutes period, the cardiac output approximately doubled returning to the control level in from 2 to 3 minutes, the heart rate increased between 10

and 15% and the systolic and diastolic pressure fell.

No detectable difference in response was noted in the dog receiving chloralose.

*Discussion.* Dibenamine in adrenolytic doses produces a significant drop in blood flow to the head or leg, but has little effect on cardiac output of the dog. The drop in blood pressure and the change in the pressure pulse are probably the result of a vasodilation in the splanchnic area which does not include the kidney.<sup>7</sup>

*Summary.* Following Dibenamine, cardiac output remained essentially unaltered in 5 of 8 dogs, peripheral blood flow decreased significantly in 16 of 24 dogs.

<sup>7</sup> Wang, C., and Nickerson, M., *Proc. Soc. Exp. Biol. and Med.*, 1949, **70**, 92.

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### 17099. Effect of an Antagonist of Pteroylglutamic Acid on the Leukemia of Ak Mice.\*

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In a previous paper<sup>1</sup> hematologic and histo-

\* This investigation was supported in part by a grant from the Division of Research Grants and Fellowship of the National Institutes of Health, U. S. Public Health Service, and, in part, by a grant from the National Vitamin Foundation.

<sup>1</sup> Weir, D. R., Heinle, R. W., and Welch, A. D., *Proc. Soc. Exp. Biol. and Med.*, 1948, **69**, 211.

logic findings in mice with an induced deficiency of pteroylglutamic acid (PGA) were described, together with a method for producing the deficiency with the aid of a crude chemical antagonist of PGA (so-called "x-methyl PGA," presumably a mixture of 7-methyl- and 9-methyl PGA).<sup>†</sup>

Following this study we became interested

in the influence of this deficiency on the transplanted leukemia of Ak mice. About 75% of this strain develop spontaneous leukemia after the age of 6 months. The intravenous injection of leukemic cells from diseased older animals produces progressive leukemia in 100% of younger animals.<sup>2</sup> It was originally planned to make the transplant after the deficiency, as evidenced by leucopenia, had been established. This approach was found to be unsuitable, probably because the Ak strain is not hardy enough to survive the dietary regime for the preliminary 6 to 8 weeks required for the development of the deficiency, together with the additional time necessary for the growth of the transplanted material. Accordingly, it was decided to transplant the leukemic cells about 15 days after the start of the experiment.

In a preliminary experiment there had been some indication that the parenteral administration of the crude synthetic antagonist of PGA (Lederle N-67A)<sup>†</sup> might be more effective than its oral administration. Accordingly, for comparative study a microbiologically assayed solution of partially purified "x-methyl PGA" was used.<sup>‡</sup> The preliminary work also indicated the desirability of pair-feeding the animals in an attempt to rule out the possible effects of inanition as a result of diminished intake of PGA.

*Methods.* Weanling Ak mice weighing with-

<sup>†</sup> The crude antagonist was prepared by Dr. M. E. Hultquist and Dr. J. M. Smith, Jr., Calco Chemical Company, by reacting 2,4,5-triamino-6-hydroxy-pyrimidine and p-amino-benzoyl-1(+)-glutamic acid with 2,3-dibromobutyraldehyde in the reaction described by Angier *et al.*, *Science*, 1946, **103**, 667. We are much indebted to Dr. T. H. Jukes of the Lederle Laboratories Division, American Cyanamid Company, who supplied us with this antagonist of PGA.

<sup>2</sup> Weir, D. R., and Heinle, R. W., *Proc. Soc. Exp. Biol. and Med.*, 1947, **66**, 268.

<sup>‡</sup> Assayed on *Streptococcus faecalis* R, each ml of the solution used contained the equivalent of 68 mg of a crystalline reference standard supplied by Dr. E. L. R. Stokstad of the Lederle Laboratories. However, it developed subsequently that this microbiologically active fraction is not responsible for the effect of the crude antagonist on animal species.

TABLE I. Results with Purified Diet and Added Oral and Parenteral Supplements.

Group	Leukemic animals (see text)	Supplements to purified diet	Survival after transplanting			Leucocytes		"Blasts"		Histological findings		
			Mean days	Max. days	Min. days	Max. daily mean, per cmm	Max. single count, per cmm	Max. daily mean, per cmm	Liver	Spleen	Marrow	
1	7	Parent. antagonist	46	71	27	38,000	55,000	1026	++	+++	+++	
2	4	Oral antagonist Parent. antagonist Parent. PGA	38	73	22	34,500	55,000	1380	+++	+++	+++	
3	4	Oral antagonist Parent. antagonist	53	77	35	14,750	27,000	140	0	+	++	
4	4	Oral antagonist Parent. Locke's sol.	45	63	19	17,000	23,000	97	0	0	++	
Controls	5	Normal diet No supplement	19	23	16	92,000	108,000	4600	++++	++++	++++	

in 5 g of each other were arranged in 4 groups of 10 animals each and one group of 5 animals (Table I). The composition of the purified diet has been given previously.<sup>1</sup> In this experiment the diet included 1% succinylsulfathiazole from the beginning. All groups were maintained on this diet except the controls. For Groups 2, 3, and 4, 0.5% powdered antagonist was added to the diet. On the 13th day parenteral therapy was started. One cc of the antagonist solution was given daily intraperitoneally to Groups 1, 2, and 3; 20  $\mu$ g of PGA in 0.2 ml of Locke's solution was given daily subcutaneously to Group 2; and 1 cc of Locke's solution was given daily intraperitoneally to Group 4. On the 16th day all animals were transplanted. The possibility that the potency of the cell suspension might deteriorate during the time of inoculation of a large group of animals was taken into account. Animals from the various groups were taken in rotation so that no one entire group was inoculated either at the beginning or end of the transplanting period.

The animals of groups 2 and 4 were fed the same amount of diet as that consumed on the previous day by the animals of Group 3 (thus, animals No. 11 and No. 31 were fed the amount of food consumed by animal No. 21 on the previous day). Twice weekly all animals were weighed and total and differential leucocyte counts were done. At death, sections of liver, spleen, and bone marrow were prepared for histologic studies.

**Results.** As in the preliminary experiments it was found that the Ak animals do not survive well under these forms of treatment; many mice died before the leukemia could have developed. Others died during the period when the controls showed florid leukemia, but did not themselves show any evidence of the disease, either by blood counts or by gross or microscopic pathologic examination. Therefore, it was considered justifiable to eliminate from consideration those animals which died early or which did not show any evidence of leukemia at any time. In Group 1, 7 animals remained and in each of Groups 2, 3, and 4, 4 animals remained. All 5 of the controls were dead of leukemia within 23 days after transplanting. Although a reliable demonstration

of the effect of the regime on time of survival was prevented by the early mortality of many of the experimental animals, other findings are of considerable interest. The results of the experiment are shown in Table I.

The control animals developed the typical picture of the transplanted disease; all had very high leucocyte counts with blast cells in the peripheral blood, and histologic sections showed extensive leukemic infiltration in the liver, spleen, and marrow. Although all controls were dead within 23 days, the mean survival time of the experimental groups was about 46 days. Control animals with transplanted Ak leukemia almost invariably die within a few days of each other, a circumstance which obtained in this experiment. In our experience the interval between inoculation and death may vary in different experiments, due probably to variation in the donors, but in any one experiment the time of survival of various individuals differs only by a few days. Accordingly, the apparent increase in the mean survival time in the experimental groups was considered to be encouraging in import.

The animals in Group 4 which received the diet and oral antagonist showed the maximal alteration of the leukemic process. The white blood cell counts never rose above the maximal normal of 24,000 per cmm and only small numbers of blast cells appeared in the peripheral blood. Histologically the livers and spleens of these mice showed no leukemic infiltration and the marrows showed only moderate infiltration. Essentially the same picture was seen in Group 3, indicating that the action of the oral antagonist was not significantly altered by the parenteral preparation. This conclusion was supported by the finding that the animals in Group 1, which received the parenteral antagonist without oral antagonist, showed a moderate increase in white blood counts, moderate numbers of blasts, and histologic findings which differed very little from the controls. The animals in Group 2 received the diet with both oral and parenteral antagonist, supplemented with parenterally administered PGA. It had been calculated that a daily dose of 20  $\mu$ g of PGA would combat all effects of the antagonist. Unfortunately this was not true. However, the

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			Mean days	Max. days	Min. days	Max. daily mean, per cmm	Max. single count, per cmm	Max. daily mean, per cmm	Liver	Spleen	Marrow	
1	7	Parent, antagonist	46	71	27	38,000	55,000	1026		++	+++	+++
2	4	Oral antagonist Parent, antagonist Parent, PGA	38	73	22	34,500	55,000	1380		+++	+++	+++
3	4	Oral antagonist Parent, antagonist	53	77	35	14,750	27,000	140		0	+	++
4	4	Oral antagonist Parent, Locke's sol.	45	63	19	17,000	23,000	97		0	0	++
Controls	5	Normal diet No supplement	19	23	16	92,000	108,000	4600		++++	++++	++++

technic did not rule out inanition as the determining factor.

If a profound, generalized deficiency of PGA were the determining factor one might expect some evidence for this in the leucocyte picture of the peripheral blood of the experimental animals at the time the controls had florid leukemia. However, no such evidence was found. In this experiment the leucocyte counts were not low at that time and in a preliminary experiment, run under similar conditions, there were no changes in either the white or red blood cell counts. Also, it has been shown in previous experiments<sup>1</sup> that hematologic and histologic evidence of PGA-deficiency do not develop until the animals have been on a purified diet containing succinylsulfathiazole and antagonist for 50 to 60 days. The rate at which PGA-deficiency might develop without the purified diet, as in Part II, has not yet been determined. Of course these findings do not mean that a selective or relative deficiency may not exist. It seems likely that leukemic cells may require a much more abundant supply of PGA than do normal cells.<sup>2</sup> A moderate decrease in available PGA, not enough to produce leucopenia in a normal animal, might be sufficient to decrease the rate of development of leukemic cells. On the other hand, except for the blasts, the leucocytes in the peripheral blood have the appearance of normal cells and merely exist in greater numbers. Perhaps the relative deficiency acts by limiting the excessive production of these essentially normal cells, rather than by interfering with the metabolism of abnormal cells. This reasoning may apply to the cells in the peripheral blood, but does not necessarily explain the marked reduction or complete absence of leukemic infiltration in the liver, spleen, and marrow of the experimental animals in Part I. The infiltrating cells are definitely abnormal as are the blasts of the peripheral blood.

If PGA-deficiency were not the sole explanation for these findings, it is conceivable that an active substance other than the antagonists of PGA may be present in the crude

antagonist. However, all growth experiments have indicated that the effects of this material are reversed completely by synthetic PGA.<sup>3,4</sup> Further experiments designed to determine whether PGA-deficiency can account completely for the observed inhibition of the leukemic process are now in progress. These experiments have shown that the leukemic process in mice can be controlled partially by methods and substances now available. Life is prolonged and the severity of the disease is reduced. It is apparent that with the techniques so far devised the effects are not of permanent value and the effect on the normal metabolic activity of the animal can be severe. If a profound deficiency of PGA is produced, the leukemia may be controlled, but the treatment eventually is as fatal as the disease. It is logical to expect that this might be true, for it may be assumed that PGA is an essential metabolite utilized in the manufacture of many types of cells and the requirement for the formation of abnormal leucocytes, though larger, probably is not inordinately greater than that for normal cell formation. That the animals of Part I, given a purified diet, developed a deficiency of PGA in the terminal state, is certain. However, additional studies will be required to prove that here also a deficiency of PGA was the only factor operating in the early stage of the experiment when leukemia was suppressed in the experimental groups while the controls had developed florid leukemia. It is our hope that a regimen may be worked out which will provide for selective inhibition of the leukemic process without too seriously impairing the processes essential to the life of normal cells.

**Summary.** The transplanted leukemia of Ak mice can be partially controlled either by a pteroylglutamic acid-free diet, combined with succinylsulfathiazole and a crude antagonist of pteroylglutamic acid, or by the addition of large amounts of the crude antagonist to an unpurified "normal mouse diet." Life is prolonged and the severity of the disease is reduced.

<sup>4</sup> Franklin, A. L., Stokstad, E. L. R., Bell, M., and Jukes, T. H., *J. Biol. Chem.*, 1947, **169**, 427.

<sup>3</sup> Bethell, F. H., and Swendseid, M. E., *J. Clin. Invest.*, 1946, **25**, 917.



TABLE II.  
Results with Normal Mouse Diet and Antagonist Added in Excess.

Group	No. of animals	Diet supplement	Leucocytes					
			Survival after transplanting			M-x. daily mean, per cmm ×1000	Max. single count, per cmm ×1000	“Blasts” max. daily mean, per cmm
			Mean days	Max. days	Min. days			
1	8	No antagonist	16.1	21	14	82	127	1,800
2	8	2% PGA-antagonist	39.0	72	31	45	88	4,200
3	8	4% “ ”	57.0	74	41	22	32	1,600

dose was sufficient to allow the development of moderately high white counts with moderate numbers of blasts and histological changes which could not be distinguished from the controls.

Within each group the histologic changes were surprisingly uniform in the individual animals. The plus values given in Table I apply to each individual animal and are not means for the group.

In all the experimental groups growth was arrested and there was a steady loss of weight. In this respect none of the groups differed from the others.

Because of the high mortality of the experimental animals on the purified diet, as described in Part I of this experiment, the effect of the PGA-antagonist, when added to an unpurified diet known to be nutritionally adequate for the mouse (Purina dog chow), was studied.

**Methods.** In preliminary experiments, PGA-antagonist at levels of 0.5% and 1.0% had had some effect on the progress of the leukemia but not enough to be statistically significant. Accordingly, 24 Ak mice, weighing within 5 g of each other, were arranged in 3 groups of 8 animals each and were fed Purina dog chow ground into a coarse powder. Group 1 (controls) was given no supplement, while Group 2 and Group 3 were given the antagonist at levels of 2 and 4%, respectively. The animals of Groups 1 and 2 were fed that amount of diet consumed by Group 3 on the previous day. Twice weekly all animals were weighed and total and differential leucocyte counts were done. On the fifteenth day all animals were transplanted. At autopsy, sections of liver, spleen, and bone marrow were made for histologic study.

**Results.** In contrast to the experience described in Part I, the dietary regimen of Part II was very well tolerated by all animals. None of the animals died in the preliminary stages and all developed evidence of leukemia before death. The results are given in Table II. With 2% antagonist the mean survival time was more than doubled, and with 4% it was more than tripled. In neither of the supplemented groups did an animal expire until at least 10 days after the last control animal had died.

The peripheral blood of the experimental animals showed more evidence of leukemia than was seen in the experimental animals on the purified diet of Part I. Even with PGA-antagonist at a level of 4% many blast forms appeared although only 2 total leucocyte counts were above 24,000, the maximal normal.

In contrast to the animals on the deficiency regimen of Part I, the histologic findings in the experimental animals of Part II were indistinguishable from those of the controls. Infiltration of all organs was extensive. Very probably this infiltration occurred later and more slowly in the experimental animals as compared to the controls.

There was a moderate loss of weight in some animals; others gained. The mean loss was considerably less than that shown by the animals on the deficiency regimen.

**Discussion.** Several factors might be considered as having contributed to the experimental results; among these are: 1) simple inanition, 2) deficiency of PGA, 3) an effect of one or more substances in the crude antagonist. Inanition might be seriously considered, if the increased survival times alone were taken into account, and if the paired feeding

TABLE I.  
A. Effect of Hemorrhage on the Blood Count of the Rat.\*

	A. Effect of hemorrhage on the blood count of the mice												
	Lymphocytes in %				Neutrophils in %				Neutrophils				% of red blood cells at 60'
	0'	20'	40'	60'	0'	20'	40'	60'	0'	20'	40'	60'	
Normal	100	85	78	67	100	99	84	80	100	110	114	121	94
Adrenodenedullated	100	91	95	92	100	83	92	86	100	95	100	96	96
Normal	100	76	55	46	100	88	84	72	100	114	127	131	101
Adrenodenedullated	100	91	84	82	100	101	100	103	100	110	116	116	98

\* Each group consisted of 10 rats which were fasted 10 hours prior to the experiment.

† Probability coefficient applied to the lymphocytes in the 2 groups at 60 minutes.

II. *The effect of "rage" on the lymphocyte count.* Normal and adrenodemedullated rats were given a slight electroshock applied to both ears for 0.4 second. The rats reacted strongly to the stimulus with struggling and vocalization but were quiet a few minutes later. The effect on the lymphocytes was similar to that described in the first group but the difference in the reaction between normal and adrenodemedullated rats was even greater ( $P < 0.001$ ). The change in the neutrophil count was again much less than in that of the lymphocytes, leading to a lymphopenia and a relative neutrophilia as on injection of adrenalin. No changes occurred in the neutrophils of the demedullated group and the fall in the lymphocytes was very slight. The red cell count before and 60 minutes after the "rage" reaction showed clearly that the changes in the lymphocytes of normal rats in conditions of "rage" are not due to a dilution of the blood.

III. *The action of hypoglycemia on the lymphocyte count.* A series of experiments listed in Table II was performed to determine the action of insulin hypoglycemia on the lymphocyte count of normal and adrenodemedullated rats. Since the latter are more sensitive to insulin than normal rats the experimental conditions had to be slightly modified for the two groups in order to obtain similar degrees of hypoglycemia. Table II shows that this was accomplished by varying the time interval of insulin injections and the duration of fasting. If both groups (normal and demedullated rats) were given 2 successive injections of 0.5 unit/kilo insulin i.p. at an interval of 20 minutes the demedullated rats were in coma at about 30 to 40 minutes but the normal rats at 60 minutes. By delaying the second injection by 10 minutes the demedullated group went into coma at about the same time as the unoperated group. As the Table shows the effect of insulin on blood sugar was very similar in groups A (normal) and D (demedullated); likewise the mild hypoglycemic effects in groups C and E were about the same. In the groups A and D in which each animal was in coma at 60 minutes the average lymphocyte count was 60 and 70.2% respectively of the preexperimental level in the normal and the

## 17100. Lymphopenia and the Secretion of Adrenalin.\*

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It was shown in a preceding paper<sup>1</sup> that the injection of adrenalin in doses of from 0.05 to 1  $\gamma$ /100 g significantly lowers the lymphocyte count of the rat while the number of neutrophils is reduced to a lesser extent. This result (lymphopenia and a relative neutrophilia<sup>1</sup>) is not seen in adrenalectomized animals. The great sensitivity of this reaction makes it probable that it may occur under conditions involving not the injection but the secretion of adrenalin. Since the question of increased adrenalin secretion in physiological conditions is still controversial (See Rogoff,<sup>2</sup> for the older literature see Gellhorn<sup>3</sup>) it was decided to investigate the action of hemorrhage, emotional excitement and insulin hypoglycemia on the lymphocyte count. Elmadjian and Pincus<sup>4</sup> showed already that struggle (excitement) and exposure to cold resulted in lymphopenia and that this effect was absent in adrenalectomized rats. However the experiments of these authors did not deal with the question whether the secretion of adrenalin formed a necessary link in the chain of events leading to lymphopenia under conditions of struggle, etc. The present work was undertaken to fill this gap.

**Method.** The experiments were performed on adult Sprague-Dawley rats (weight 150 to 200 g) after a 16 hour fast unless otherwise stated. Blood samples were taken from the tail blood and the lymphocytes were deter-

mined before and 20, 40 and 60 minutes after the experimental procedure. Three procedures were used. The first consisted in hemorrhage (1 cc blood/100 g weight removed from the tail veins); the second involved emotional excitement induced by a slight electric shock which elicited a "rage" reaction but did not produce a convulsion. As a third procedure insulin<sup>†</sup> was injected intraperitoneally and the effect of varying degrees of hypoglycemia was studied. The blood sugar was determined as in previous papers.<sup>5</sup> The experiments were performed on normal and adrenomedullated rats. The latter were used not earlier than 10 to 14 days after operation and only after a test with adrenalin 0.1  $\gamma$ /100 g i.p. had resulted in a marked lymphopenia. If this effect was not clear cut the animal was either discarded or used after a second adrenalin test taken one or two weeks later had proved the responsiveness of the adrenal cortex to injected adrenalin.

**Results. I. The effect of hemorrhage on the lymphocyte count.** Table I shows the percentage changes in lymphocytes and polynuclear leucocytes following hemorrhage. In 60 minutes the former fell to 66.9% of the control value in normal rats whereas adrenomedullated animals showed only an insignificant fall (to 91.5%). This difference is statistically significant ( $P < 0.01$ ). The polynuclear cells fell only slightly. As the red cell count indicates the difference existing between the normal and adrenomedullated rats is not accounted for by differences in the dilution of the blood and must therefore be attributed to a reaction dependent on the secretion of adrenalin. The fact that the reaction observed in normal rats has the same characteristics (lymphopenia and relative neutrophilia) as seen after injection of adrenalin supports this conclusion.

\* Aided by a grant from the Office of Naval Research.

1 Gellhorn, E., and Frank, S., *Proc. Soc. Exp. Biol. and Med.*, 1948, **69**, 426.

† Through an oversight this effect was described in the preceding paper as lymphopenia and neutrophilia instead of lymphopenia and relative neutrophilia.

2 Rogoff, J. M., *J. Genetic Psychol.*, 1945, **32**, 249.

3 Gellhorn, E., *Autonomic Regulations*, New York, 1943.

4 Elmadjian, F., and Pincus, G., *Endocrinology*, 1945, **37**, 47.

† Kindly supplied by the Eli Lilly and Co.

5 Safford, H., and Gellhorn, E., *Proc. Soc. Exp. Biol. and Med.*, 1945, **60**, 98.

demedullated group. The statistical evaluation shows that  $P$  is  $< 0.05$  suggesting probable significance. As the hypoglycemic effect diminishes from A to C in the normal group (the final, 60 minutes—blood sugar being 28, 43 and 50 mg % respectively) the lymphopenia becomes progressively less (60% in group A, 70% in B and 75% in C). Comparison of A to C on the basis of the lymphocyte count at 60 minutes gives  $P < 0.01$  indicating that the difference is statistically significant. On the other hand similar differences in hypoglycemia in the demedullated group (D and E) do not lead to statistically significant differences in lymphocyte count. These data suggest that in hypoglycemia the increased secretion of adrenalin contributes to lymphopenia. However further control experiments appeared in order. Therefore a final group of insulin tests was performed on adrenalectomized rats in which the count of red cells and lymphocytes, and the blood sugar were determined. As Table II shows adrenalectomized rats show only a very slight fall in lymphocytes and no characteristic changes in neutrophils. In view of the fact that the lymphocytes fell more in demedullated than in adrenalectomized animals subjected to insulin coma it is suggested that in addition to the lymphopenia initiated by the increased secretion of adrenalin some other effect of low blood sugar on the adrenal cortex occurs which leads to lymphopenia without the intervention of functional changes in the secretion of adrenalin. Here again the red blood count remained unchanged.

Our investigations show that hemorrhage, general ("emotional") excitement and, to a lesser extent, insulin hypoglycemia lead to a lymphopenia and a relative neutrophilia in normal but not in adrenodemedullated rats. As was shown previously similar changes may be induced by the injection of adrenalin. Since hemorrhage, general excitation and injection of adrenalin no longer cause these effects in adrenalectomized rats it must be concluded that emotional excitement and hemorrhage lead to an increased secretion of adrenalin which indirectly, probably through a decrease in the level of cortical hormones in blood and

tissues (Sayers and Sayers,<sup>6</sup> Gellhorn and Frank), leads to a discharge of adrenocortical hormones resulting in lymphopenia. Insulin hypoglycemia causes a similar reaction but our experiment suggests an additional action of hypoglycemia on the hypophyseal-adrenocortical system since hypoglycemia acts much more on the lymphocyte count of adrenodemedullated than on that of adrenalectomized rats. This action appears to be the cause of the lesser difference between adrenodemedullated and normal rats under conditions of hypoglycemic coma than was found between these groups of animals subjected to hemorrhage and excitement. Observations by Parsons and collaborators<sup>7</sup> on the action of insulin hypoglycemia and injection of adrenalin on the blood count of schizophrenics agree with our present and previous experiments except for the fact that in these experiments lymphopenia is associated with an absolute neutrophilia whereas in our work on rats injection or secretion of adrenalin leads to lymphopenia with relative neutrophilia. However, this difference is probably accounted for by the fact that adrenalin in large doses causes lymphopenia and neutrophilia (Hungerford).<sup>8</sup>

*Summary.* Hemorrhage (1 cc/100 g body weight) and general excitement (induced by the action of a subconvulsive electrical shock) cause lymphopenia and relative neutrophilia in normal but not in adrenodemedullated rats. Insulin hypoglycemia leading to coma induces a greater lymphopenia in normal than in adrenodemedullated animals. It is concluded that hemorrhage, general excitement and insulin coma cause an increased secretion of adrenalin followed by an increased discharge of adrenocortical hormones. Hypoglycemia seems to induce some increased adrenocortical secretion even in the absence of the adrenal medulla.

<sup>6</sup> Sayers, G., and Sayers, M. A., *Endocrinology*, 1947, **40**, 265.

<sup>7</sup> Parsons, E. H., Gildea, E. F., Ronzoni, E., and Hulbert, S. Z., *Am. J. Psychiat.*, 1949, **105**, 573.

<sup>8</sup> Hungerford, G. F., *Proc. Soc. Exp. Biol. and Med.*, 1949, **70**, 356.

TABLE II.  
A. Effect of Insulin Hypoglycemia on the Blood Count of the Rat. Dose 0.5  $\mu$ /kg.

Group	Hrs fasted	Dose	Blood sugar at 60'	% of lymphocytes						% of neutrophils						Neutrophils									
				0'	20'	40'	60'	0'	20'	40'	60'	0'	20'	40'	60'	0'	20'	40'	60'						
A. Normal*	36	at 0' and 20'	28.3 $\pm$ 8.48	100	83	73	60	100	91	83	76	100	112	122	136	100	112	122	100	112	122	100	112	122	136
B. "†	16	at 0' and 20'	43.3 $\pm$ 6.39	100	83	77	70	100	89	84	83	100	107	115	123	100	107	115	100	107	115	100	107	115	123
C. "†	16	at 0'	50.2 $\pm$ 4.96	100	89	83	75	100	93	83	80	100	106	106	112	100	106	106	100	106	106	100	106	106	112
D. Adrenomedullated	24	at 0' and 30'	31.8 $\pm$ 9.6	100	82	75	72	100	94	84	87	100	101	102	107	100	101	102	100	101	102	100	101	102	107
E. "†	16	at 0' and 20'	46.4 $\pm$ 14.97	100	92	85	79	100	92	85	88	100	98	99	113	100	98	99	100	98	99	100	98	99	113

\* Group A and D consisted of 11 animals.

† Group B, C, and E consisted of 10 animals.

B. Significance of the Changes in Lymphocytes.

Groups compared	P*
A, C	<.01
A, D	<.05
D, E	<.2
A, B	<.05
C, D	<.9
C, E	.5

\* Probability coefficient applied to lymphocytes in the two groups at 60'.

C. Effect of Insulin Hypoglycemia on the Blood Count of the Adrenalectomized Rat.\*

Blood sugar at 60' (mg%)	% of lymphocytes						Neutrophils						% red blood cells at 60'					
	0'			20'			0'			20'			0'			20'		
	at 60'			at 60'			at 60'			at 60'			at 60'			at 60'		
31 $\pm$ 2.9	100	101	98	90	100	101	95	107	100	99	98	111	100	98	111	100	98	111

\* Insulin 0.2  $\mu$ /kg was injected in 10 rats fasted for 16 hours and resulted in deep coma.

demedullated group. The statistical evaluation shows that  $P$  is  $< 0.05$  suggesting probable significance. As the hypoglycemic effect diminishes from A to C in the normal group (the final, 60 minutes—blood sugar being 28, 43 and 50 mg % respectively) the lymphopenia becomes progressively less (60% in group A, 70% in B and 75% in C). Comparison of A to C on the basis of the lymphocyte count at 60 minutes gives  $P < 0.01$  indicating that the difference is statistically significant. On the other hand similar differences in hypoglycemia in the demedullated group (D and E) do not lead to statistically significant differences in lymphocyte count. These data suggest that in hypoglycemia the increased secretion of adrenalin contributes to lymphopenia. However further control experiments appeared in order. Therefore a final group of insulin tests was performed on adrenalectomized rats in which the count of red cells and lymphocytes, and the blood sugar were determined. As Table II shows adrenalectomized rats show only a very slight fall in lymphocytes and no characteristic changes in neutrophils. In view of the fact that the lymphocytes fell more in demedullated than in adrenalectomized animals subjected to insulin coma it is suggested that in addition to the lymphopenia initiated by the increased secretion of adrenalin some other effect of low blood sugar on the adrenal cortex occurs which leads to lymphopenia without the intervention of functional changes in the secretion of adrenalin. Here again the red blood count remained unchanged.

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**Summary.** Hemorrhage (1 cc/100 g body weight) and general excitement (induced by the action of a subconvulsive electrical shock) cause lymphopenia and relative neutrophilia in normal but not in adrenodemedullated rats. Insulin hypoglycemia leading to coma induces a greater lymphopenia in normal than in adrenodemedullated animals. It is concluded that hemorrhage, general excitement and insulin coma cause an increased secretion of adrenalin followed by an increased discharge of adrenocortical hormones. Hypoglycemia seems to induce some increased adrenocortical secretion even in the absence of the adrenal medulla.

<sup>6</sup> Sayers, G., and Sayers, M. A., *Endocrinology*, 1947, **40**, 265.

<sup>7</sup> Parsons, E. H., Gildea, E. F., Ronzoni, E., and Hulbert, S. Z., *Am. J. Psychiat.*, 1949, **105**, 573.

<sup>8</sup> Hungerford, G. F., *Proc. Soc. Exp. Biol. and Med.*, 1949, **70**, 356.

# 17101 P. Interaction of Swine Influenza Virus and Egg-White Inhibitor of Virus Hemagglutination.\*

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The interaction of swine influenza virus and egg-white (EW) inhibitor of virus hemagglutination resembles an enzymatic reaction with the virus acting as enzyme and the inhibitor as substrate.<sup>1</sup> Attempts to follow the kinetics of inhibitor inactivation by virus have revealed that the inhibitor present during the intermediate period of the reaction differs in behavior from untreated inhibitor. This observation and its possible significance are the subjects of the present report.

**Materials and Methods.** The swine influenza virus was the purified preparation SF, previously described,<sup>1</sup> which contained about 7,200 hemagglutinating doses (HD) and 0.234 mg N per ml. The preparation of semipurified inhibitor, A200 PII EI, was obtained by precipitating EW with 7 volumes 0.1 M  $\text{KH}_2\text{PO}_4$  and extracting the precipitate, after washing, with 0.06 M phosphate buffer at pH 7.2.<sup>2</sup> The extract contained 120  $\gamma$  N per ml and was about 30 times as active as EW on a N basis. The residual inhibitory activity of virus-inhibitor mixtures was titrated against 4 HD heated swine virus (53°C, 30 minutes) by the constant virus-varying inhibitor method.<sup>2</sup> Preliminary to titration, the virus-inhibitor mixtures were immersed in boiling water for 2 minutes to destroy the hemagglutinating activity of the virus; the activity of the inhibitor was not significantly affected by this treatment. Buffered saline, consisting of 0.81% NaCl and 0.005 M phosphate at pH 7.3, was the diluent throughout.

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1 Lanni, F., and Beard, J. W., *Proc. Soc. Exp. Biol. and Med.*, 1948, **68**, 442.

2 Lanni, F., Sharp, D. G., Eckert, E. A., Dillon, E. S., Beard, D., and Beard, J. W., *J. Biol. Chem.*, in press.

**Experimental.** In a typical experiment, mixtures of equal volumes of 1:5 inhibitor and 1:200 SF were incubated for varying periods at room temperature (27°C), immersed in boiling water, cooled, and titrated for residual inhibitory activity with virus heated at 53°C; inhibitor controls devoid of virus were included. The complete titration data are shown in Fig. 1, in which both the conventional designations, 0 to +++++, denoting minimal to maximal hemagglutination, and the corresponding chicken red blood cell concentrations in the reference red-cell suspensions are used as ordinates. In the present experiment, the designation +++± corresponds to zero inhibition. From the results it is evident that virus-treated inhibitor cannot properly be described as diluted untreated inhibitor, since the inhibition curves cannot be superimposed by translation along the axis of inhibitor dilution; therefore, no simple kinetic analysis is possible. Hirst<sup>3</sup> has re-

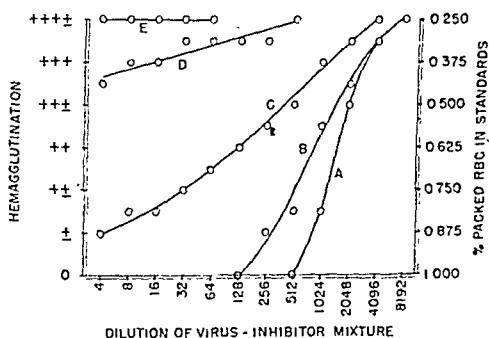


FIG. 1.

Inhibitory activity of purified EW inhibitor (A200 PII EI) after incubation with purified swine influenza virus (SF) for varying periods at 27°C, as tested with heated (53°C, 30 minutes) virus. Period of incubation: curve A, 0 minutes; B, 3 minutes; C, 10 minutes; D, 30 minutes; E, 100 minutes. Curve A applies also to untreated inhibitor control and to inhibitor control heated in boiling water for 2 minutes.

<sup>3</sup> Hirst, G. K., *J. Exp. Med.*, 1948, **87**, 315.

ported a similar result with normal serum inhibitor.

Experiments to clarify the origin of the progressive decrease in slope induced by virus (Fig. 1) have shown that (a) the slope effect is not attributable to the concentration of impurities (cf. 2) or to the accumulation of non-inhibitory reaction products; and (b) the inhibitor characterized by shallow slope does not sediment appreciably in centrifugal fields sufficient for sedimenting virus.

**Discussion.** The slope of the inhibition curves (Fig. 1) in the region of transition from zero to maximal agglutination may be tentatively interpreted in terms of the firmness of attachment of inhibitor to the particles of the *titrating* virus. Accordingly, virus-treated inhibitor may be described as a "weak" inhibitor in comparison with untreated inhibitor.

Available evidence suggests that the weak inhibitor is a free inhibitor, unattached to virus. Such an inhibitor could pre-exist in a heterogeneous inhibitor population and could be made manifest through a preferential action of virus against strong inhibitor. An attractive alternative is that the weak inhibitor is

the product of a progressive, rather than all-or-none, action of virus. If the latter explanation should be correct, the demonstration of a free, weak inhibitor, intermediate between fully active and inactive inhibitor, would provide strong support for the enzymatic hypothesis of inhibitor inactivation by virus. The possibility that the action of virus is not all-or-none has been previously suggested by Burnet and collaborators<sup>4,5</sup> from related, but somewhat more complicated, experiments.

**Summary.** During the interaction of swine influenza virus and egg-white inhibitor of hemagglutination, there appears a weak inhibitor, which has titrating properties different from those of untreated inhibitor. This result renders impossible a simple kinetic treatment of the data. The significance of the weak inhibitor for the mechanism of virus-inhibitor interaction is discussed.

<sup>4</sup> Burnet, F. M., *Austr. J. Exp. Biol. and Med. Sci.*, 1948, **26**, 389.

<sup>5</sup> Anderson, S. G., Burnet, F. M., Fazekas de St. Groth, S., McCrea, J. F., and Stone, J. D., *Austr. J. Exp. Biol. and Med. Sci.*, 1948, **26**, 403.

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## 17102. Role of Adrenal in Uptake of $I^{131}$ by the Thyroid Following Parenteral Administration of Epinephrine.

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In a previous paper<sup>1</sup> we reported that the parenteral injection of epinephrine into both the intact and the totally thyroidectomized dog resulted in an increase in secretion of thyrotropin from the adenohypophysis. In the intact dog this was evidenced by the development of hyperplastic changes in the thyroid following the daily injection of adrenalin-in-oil. Serum obtained from similarly treated totally thyroidectomized animals, when injected subcutaneously into young guinea pigs not exceeding 200 g in weight, resulted in hyperplastic changes in the thy-

roids of the treated guinea pigs.

It was further reported that the increase in circulating thyrotropic factor resulting from the injection of epinephrine in totally thyroidectomized dogs reached its peak approximately 4 to 6 days following the beginning of treatment, and thereafter began to diminish despite the continued injection of epinephrine.

The present report is concerned with a study of the role of the adrenals in the above described phenomena. In place of the biological assay method previously employed for the determination of circulating thyrotropin, we used the percentage uptake of parentally administered  $I^{131}$  as an index of thyroid activity. This technique has the relative ad-

<sup>1</sup> Soffer, L. J., Volterra, M., Gabrilove, J. L., Pollack, A., and Jacobs, M., *Proc. Soc. Exp. Biol. and Med.*, 1947, **64**, 446.



vantages of simplicity and a higher degree of accuracy over the biological assay method.

**Methods.** Male rats of the Wistar strain (Carworth Farms) weighing approximately 125 g were employed. They were placed on a low iodine, minimally goitrogenic (Steenboch) diet\* for at least 3 weeks before being used experimentally in order to insure an adequate uptake by the thyroid gland of  $I^{131}$ .<sup>2</sup> In the adrenalectomy experiments, rats of the Sprague-Dawley strain were also employed because of the reported lesser frequency of accessory adrenal tissue.

The experiments were planned to study the 24-hour percentage uptake of parenterally administered  $I^{131}$  by the thyroid gland after the administration of epinephrine in the intact and in the adrenalectomized animals. Some preliminary studies were conducted to determine the influence of the daily injection of 17-hydroxy-11 dehydrocorticosterone† upon the response of the bilaterally adrenalectomized animals.

All experiments were conducted with litter mates acting as controls. The animals in each experimental group were subjected to the same diet and environment and for the same duration of time, although the period of time that the respective groups were kept on the low iodine diet varied from experiment to experiment. In no instance, however, was such a period less than 3 weeks.

In the intact animals epinephrine was administered twice a day at 9:00 A.M. and 5:00 P.M., for 3, 7, and 32 days. Each dose consisted of 0.1 cc of 1:1000 aqueous epinephrine and 0.1 cc of epinephrine-in-oil, 1:500, injected subcutaneously in separate sites. The morning following the last injection of epinephrine (approximately 17 hours later) 2 microcuries of  $I^{131}$  was administered

intraperitoneally. Twenty-four hours following the administration of the radioactive iodine the animals were killed by the intraperitoneal administration of Nembutal, and the thyroid glands removed.

In the experiments involving the adrenalectomized animals, the rats were bilaterally adrenalectomized after being on the low iodine diet for a period of not less than 3 weeks. At least one week was permitted to elapse following adrenalectomy before the injections of epinephrine were started. The adrenalectomized animals were maintained during the experimental period by the addition of sodium chloride to the drinking water, in isotonic concentration. Epinephrine was administered twice a day for 3 days in the same dosage given to the intact animals. In one experiment, one group of adrenalectomized animals received simultaneous injections of both epinephrine and 17-hydroxy-11 dehydrocorticosterone. The latter was administered subcutaneously in a dosage of 2 mg daily for 3 days.

The radioactive iodine was administered and the animals sacrificed in the same manner described for the intact animals. The thyroid glands were carefully dissected free of connective tissue weighed on a torsion balance, and placed in 2 cc of 1% sodium hydroxide. Digestion was carried out over a steam bath. The solution was diluted to 4 cc, an aliquot removed, placed in a metal cap, and allowed to dry. The  $\beta$  radiation of the sample was measured with a Geiger counter employing the original solution of  $I^{131}$  as a standard for calculation of the percentage uptake.

**Results.** In Table I are presented the results obtained in the intact animals. It will be noted that in each experimental group the percentage uptake of  $I^{131}$  is considerably greater in the control group than in the comparable group treated with epinephrine. The percentage uptake of  $I^{131}$  of the treated group varied from 45.3 to 66.8% of that of the untreated controls.

The injection of epinephrine (Table II) into the adrenalectomized animals, however, resulted in an increase in the percentage uptake of  $I^{131}$  as compared to that of the

\* The Steenboch diet is as follows:

Yellow corn	76%
Wheat gluten	20%
Calcium carbonate	3%
Sodium chloride	1%
Irradiated yeast	4 g/k
Drinking water containing 0.06 $\mu$ g KI/cc	

<sup>2</sup> Rawson, R. W., Tannheimer, J. F., and Peacock, W., *Endocrinol.*, 1944, **34**, 245.

† The 17-hydroxy-11-dehydrocorticosterone was kindly supplied by the Merck Co., Rahway, N. J.

TABLE I. Normal Rats.

Treatment days with epinephrine,	Epinephrine injected				Controls				% uptake of treated animals as compared to controls %
	No. of rats	Avg body wt, g	Avg thyroid wt, mg	Avg T <sub>3</sub> uptake, %	No. of rats	Avg body wt, g	Avg thyroid wt, mg	Avg T <sub>3</sub> uptake, %	
3	5*	140	9.2 ± 0.9§	7.3 ± 1.4	6*	149	10.1 ± 1.0	16.1 ± 5.5	45.3
7	6*	162	9.8 ± 1.5	12.4 ± 5.9	5*	187	11.2 ± 1.3	26.7 ± 4.3	48.3
32	6*	147	9.9 ± 2.3	23.7 ± 6.7	6*	162	11.3 ± 1.4	35.5 ± 6.3	66.8

TABLE II.  
Electronized Rats.

		Adrenalectomized Rats.		
3	4*	136	9.5 ± 0.4	23.6 ± 1.8
3	3†	153	8.4 ± 1.0	27.6 ± 9.8
3	3‡	163	14.3 ± 0.7	28.0 ± 6.6
7-hydroxy-11-dehydrocorticosterone	3††	163	14.1 ± 1.4	17.8 ± 1.0

\* Wistar strain rats, † Sprague-Dawley rats, ‡ 2 mg daily for 3 days, § S.D.

\* Wistar strain rats, † Sprague-Dawley rats, ‡ 2 mg daily for 3 days, § S.D.

non-injected adrenalectomized controls. In these experiments the percentage uptake of the administered  $I^{131}$  in the epinephrine treated adrenalectomized rats varied from 127 to 156% of that of the untreated adrenalectomized rats. In one small group of adrenalectomized animals injected with both epinephrine and 17-hydroxy-11-dehydrocorticosterone, there occurred a reversal of this phenomenon with a marked reduction in the uptake of radioactive iodine.

**Conclusions.** The injection of epinephrine into the intact rat results in a considerable decrease in the uptake of  $I^{131}$ . This is in sharp contrast to the results obtained in similarly treated adrenalectomized rats. In this group, the injections of epinephrine after adrenalectomy is followed by a pronounced increase in the percentage uptake of radioactive iodine. These experiments would indicate that the adrenal cortex exercises an inhibitory effect on the thyrotropin stimulating action of epinephrine. The results obtained in the rat are somewhat different from those observed in the dog, in that in the latter animal the inhibitory effects of the adrenal are not manifest until after several days of treatment with epinephrine. This apparently represents a species difference, although a different technique was employed.

In one preliminary group of experiments it was found that the parenteral administration of 17-hydroxy-11-dehydrocorticosterone, one of the carbohydrate acting fractions of the adrenal cortex, inhibited the thyrotropin stimulating effect of epinephrine.

That the decrease in uptake of  $I^{131}$  in the epinephrine treated intact animals is not due to the vasoconstrictor effects of the epinephrine is evidenced by the fact that in similarly treated adrenalectomized animals the percentage uptake of  $I^{131}$  is considerably greater than that of the untreated controls. A period of 17 hours after the last injection of epinephrine was allowed to elapse before the administration of  $I^{131}$  in order to avoid any possible vasoconstrictor effects.

We wish to extend our sincere thanks to the Physics Laboratory of the Hospital for their aid with the isotope studies.

# 17103. Some Chemical and Morphological Changes Elicited in the Adrenal by Stilbestrol and LAP.

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Preliminary observations suggested that a lyophilized anterior pituitary preparation (LAP), when administered simultaneously with stilbestrol, inhibits the discharge of adrenal sudanophilic material which is normally a characteristic effect of this synthetic estrogen. Hence the following experiment was planned to study in a more comprehensive manner the chemical and morphological changes produced in the adrenal by LAP, stilbestrol and combined treatment with both these substances.

**Materials and methods.** 48 female piebald rats averaging 140 g in weight were divided into 4 groups of 12 rats each and treated as follows: Group I served as normal controls. Group II received 20 mg of LAP in 0.2 cc of saline twice daily subcutaneously. Group III was given 1 mg of stilbestrol dissolved in 0.2 cc of corn oil once daily subcutaneously. In addition this group received a saline injection on the 9th day (to equalize the injection-trauma with Group IV). Group IV was given both LAP and stilbestrol in the same manner as Groups II and III respectively. At the end of 10 days the rats were killed by ether anesthesia. The adrenal glands from 6 rats of each group were immediately removed and weighed separately to 1 mg of accuracy. One gland of each rat was then placed into 4% trichloroacetic acid for ascorbic acid determination (method of Roe and Kuether<sup>1</sup>) and the other into acetone-alcohol for cholesterol estimation (method of Sperry and Schoenheimer, modification of Sperry<sup>2</sup>).

The adrenals of the other 6 rats of each group were used for histological study as follows:

\* This work was initiated while the author was a holder of a research fellowship from the Hoffman-LaRoche Co., Nutley, N. J.

<sup>†</sup> This work was done during the tenure of a Life Insurance Medical Research Fellowship.

<sup>1</sup> Roe, J. H., and Kuether, C. A., *J. Biol. Chem.*, 1943, **147**, 399.

<sup>2</sup> Sperry, W. M., *Am. J. Clin. Path.*, 1938, **2**, 91.

low: one was fixed in 10% formalin for 48 hours after which frozen sections were made at 10  $\mu$ . These were stained for lipids with Sudan IV and for "Plasmalogens" with the Schiff reagent (method of Feulgen and Voigt as described by Lison<sup>3</sup>). The other adrenal and thymus were fixed in Heidenhain's Suza fixative for H and E paraffin sections cut at 6  $\mu$ .

**Results.** A summary of our results is given in Table I which includes a statistical comparison of the values obtained.

**Weight changes.** As thymus and adrenal weight changes are inversely related to each other, they can be considered together. In all experimental groups there was adrenal enlargement and thymus involution. These changes were most pronounced in Group IV and least marked in Group II.

**Chemical changes.** The cholesterol and ascorbic acid values are expressed in mg/100 mg of adrenal tissue (concentration) and in mg/100 g of body weight (total content).

After LAP treatment (Group II) there is a decrease in the concentration and total content of adrenal *cholesterol*; only the decrease in concentration being significant. Stilbestrol (Group III) causes a very marked and significant decrease in both concentration and content of adrenal cholesterol. After LAP and stilbestrol (Group IV) these values were still lower, but not significantly different from the former group (Group III).

In a progressive order, LAP (Group II), stilbestrol alone (Group III) and in conjunction with LAP (Group IV) significantly decreased the adrenal *ascorbic acid* concentration. It is of interest that while the changes in the total content and concentration of cholesterol run parallel, no such parallelism is evident as regards ascorbic acid.<sup>4</sup>

<sup>3</sup> Lison, L., *Histochimie Animale*, Gauthier-Villars, Paris, 1936.

<sup>4</sup> Ludewig, S., and Chanutin, A., *Endocrinology*, 1947, **41**, 135.

TABLE I.  
Effect of LAP\* and Stilbestrol on Thymus and Adrenal Weight and Adrenal Cholesterol and Ascorbic Acid.

Group	Treatment	Thymus weight		Adrenal weight	
		Mg/100 g body	P†	Mg/100 g body	P
I	0	116 ± 22		32 ± 2.5	
II	LAP	47 ± 6.5	II-I <.01	36 ± 0.5	II-I <.01
III	Stilbestrol	56 ± 4.8	III-I <.02	79 ± 6.6	III-I <.01
IV	Stilbestrol	21.5 ± 1.5	IV-I <.01	85.9 ± 14.5	IV-I <.01
	LAP		IV-II <.01		IV-II <.01
			IV-III <.01		IV-III >.1
Adrenal cholesterol					
Group	Treatment	Mg/100 g body	P	Mg/100 mg adrenal tissue	P
I	0	.623 ± .088		2.674 ± .168	
II	LAP	.375 ± .076	II-I >.1‡	1.047 ± .231	II-I <.01
III	Stilbestrol	.300 ± .058	III-I <.02	.385 ± .087	III-I <.01
IV	Stilbestrol	.186 ± .021	IV-I <.01	.227 ± 0.15	IV-I <.01
	LAP		IV-II <.05		IV-II <.01
			IV-III <.1‡		IV-III >.1
Adrenal ascorbic acid					
Group	Treatment	Mg/100 g body	P	Mg/100 mg adrenal tissue	P
I	0	.105 ± .014		.442 ± .050	
II	LAP	.117 ± .008	II-I >.1‡	.322 ± .020	II-I <.05
III	Stilbestrol	.168 ± .027	III-I <.1‡	.222 ± .050	III-I <.02
IV	Stilbestrol	.097 ± .014	IV-I >.1‡	.122 ± .021	IV-I <.01
	LAP		IV-II >.1‡		IV-II <.01
			IV-III <.05		IV-III >.1‡

\* Lyophilized Anterior Pituitary.

† Probability of a chance occurrence of the difference between the means, from Fisher's table of T values.

‡ Difference not statistically significant.

**Histology.** As the results of Sudan and Schiff stains were in the same direction, only the former will be discussed.

The sudanophilia of the adrenal cortex was greatly increased by LAP. In contrast stilbestrol caused a marked discharge of sudanophilic granules. Curiously, combined stilbestrol and LAP treatment actually increased the cortical lipid content above normal, with a maximal concentration in the outer fasciculate.

Examination of H. and E. sections revealed a predominant hyperplasia of the adrenals with slight hyperemia in the group treated with LAP. The stilbestrol and especially the LAP and stilbestrol treated rats evidenced a degree of hyperplasia which was completely overshadowed by a marked congestion and edematous imbibition resulting in severe dis-

ruption of the glandular structure.

In the *thymus* there was nuclear pyknosis which appeared to parallel the degree of atrophy.

**Discussion.** Hypertrophy,<sup>5,6</sup> loss of sudanophilic material<sup>7-9</sup> as well as depletion of cholesterol<sup>10-12</sup> and ascorbic acid<sup>13-15</sup> followed by

<sup>5</sup> Selye, H., *Brit. J. Exp. Pathol.*, 1936, **17**, 234.

<sup>6</sup> Tepperman, J., Engel, F. L., and Long, C. N. H., *Endocrinology*, 1943, **32**, 373.

<sup>7</sup> Dalton, A. J., Dosne, C., and Selye, H., *Anat. Rec.*, 1940, Suppl. **76**, 85.

<sup>8</sup> Zwemer, R. L., *Am. J. Pathol.*, 1936, **12**, 107.

<sup>9</sup> Darrow, D. C., and Sarason, E. L., *J. Clin. Invest.*, 1944, **23**, 11.

<sup>10</sup> Popjack, G., *J. Path. and Bact.*, 1944, **50**, 485.

<sup>11</sup> Levin, L., *Endocrinology*, 1945, **37**, 34.

<sup>12</sup> Long, C. N. H., *Rec. Progr. in Horm. Res. Proc. Laur. Horm. Conf.*, 1947, **1**, 99.

a return of these substances to or above normal levels, have been repeatedly described as characteristic of the adrenal response to stress. The alterations in cholesterol, ascorbic acid and stainable lipids have been considered by Sayers and Sayers<sup>16</sup> to closely parallel each other. Indeed the observation by these workers of a simultaneous decrease in cholesterol and sudanophilic material has prompted them to identify the latter with cholesterol esters. The present study shows that it is possible to dissociate the sudanophilia from the cholesterol content of the adrenal cortex. LAP, either alone or in conjunction with stilbestrol, decreases the cholesterol but increases the sudanophilia of the adrenal. This suggests that the sudanophilic substance of the cortex is probably not cholesterol. Earlier experiments<sup>17</sup> have demonstrated that, under certain conditions, purified ACTH can cause a similar dissociation. Finally it appears from the above data that LAP, given with stilbestrol, markedly inhibits that loss of sudanophilic granules from the cortex which is characteristic of stilbestrol treatment.

<sup>13</sup> Torrance, C. C., *J. Biol. Chem.*, 1940, **132**, 575.

<sup>14</sup> Harkins, H. N., and Long, C. N. H., *Am. J. Physiol.*, 1945, **144**, 661.

<sup>15</sup> Sayers, G., and Sayers, M. A., Tsan-Ying Liang, and Long, C. N. H., *Endocrinology*, 1945, **37**, 96.

<sup>16</sup> Sayers, G., and Sayers, M. A., *Rec. Progr. in Horm. Res., Proc. Laur. Horm. Conf.*, 1948, **2**, 81.

<sup>17</sup> Constantinides, P., Fortier, C., Skelton, F. R., Timiras, P., and Selye, H., in press.

Further experiments are under way to study the effects of purified corticotrophic preparations on the adrenal changes elicited by non-specific stress.

**Summary.** The adrenal cholesterol and ascorbic acid levels were studied in relation to the stainable lipids of the gland in rats treated with a lyophilized anterior pituitary preparation (LAP), given alone or in conjunction with a synthetic folliculoid (stilbestrol):

1. In stilbestrol treated rats, an almost total depletion of stainable lipids was observed in conjunction with low cholesterol and ascorbic acid values.

2. LAP caused accumulation of sudanophilic lipids in the adrenal cortex but significantly lowered the cholesterol and ascorbic acid levels.

3. A similar dissociation of sudanophilic material from cholesterol and ascorbic acid, was even more evident when LAP was administered concurrently with stilbestrol. The effect of stilbestrol on sudanophilia was counteracted by LAP.

4. These observations show that the corticotrophic effect of folliculoids cannot be solely ascribed to the liberation of anterior pituitary corticotrophin such as is present in LAP.

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## 17104. Effect of Various Drugs on Epinephrine-Induced Pulmonary Edema in Rabbits.\*

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Large doses of epinephrine are known to cause a fulminating pulmonary edema in a number of species of animals.<sup>1-3</sup> Various

\* Parke, Davis and Co., Detroit, Mich., supplied a grant in support of this investigation, as well as the adrenergic blocking drugs employed.

<sup>1</sup> Meltzer, S. J., *Am. Med.*, 1904, **8**, 191.

<sup>2</sup> Emerson, H., *Arch. Int. Med.*, 1909, **3**, 368.

<sup>3</sup> Bovet, D., and Bovet-Nitti, F., *Structure et activité pharmacodynamique des médicaments du système nerveux végétatif*, S. Karger, Bale, 1948, p. 23.

workers have claimed that atropine,<sup>4,5</sup> hypnotics,<sup>6</sup> tissue extracts,<sup>7</sup> antihistaminics<sup>8</sup> and pituitrin<sup>9</sup> can diminish or prevent this edema formation. Furthermore, various adrenergic blocking drugs, as well as vasodilator drugs have been shown to be capable of reducing the mortality which follows toxic doses of epinephrine in mice<sup>10</sup> and rats.<sup>11,12</sup> A variety of explanations concerning the cause of pulmonary edema have been based, in part, on data relating to the effects of drugs on the toxicity of epinephrine.

The drugs which have been reported to protect against epinephrine-induced pulmonary edema vary widely in properties and several are relatively specific in action. If the claims made are true it would suggest that numerous mechanisms are involved in edema production or that the several protective drugs have a common property, which seems quite unlikely. Preliminary to studies aimed more directly at the elucidation of the mechanisms involved in the production of pulmonary edema and the control by drugs it was considered advisable to re-examine the effects of some of these drugs on epinephrine-induced pulmonary edema under standardized experimental conditions.

**Method.** Drugs were administered as indicated in Table I to male rabbits weighing between 2 and 3 kg prior to the intravenous injection, in 15 seconds, of 0.375 mg/kg of epinephrine (calculated as the base). The epinephrine solution employed (0.1%) was

TABLE I. Alteration of Pulmonary Edema and Mortality Due to Large Doses of Epinephrine in Rabbits.

	No. exp.	Mean body wt, kg	Mean lung wt, g $\pm$ s	Diff. from controls, g $\pm$ s	P*	% mortality†	Diff. from epinephrine controls	P*
Epinephrine	18	2.32	21.77 $\pm$ 7.19	—	—	61.1	—	—
Normal lung wt	12	2.59	10.58 $\pm$ 1.41	—	—	—	—	—
Control Groups.								
Pentobarbital Na, 25 mg/kg I.V.	6	2.52	22.34 $\pm$ 6.36	+ 0.57 $\pm$ 3.3	> 0.5	66	+ 4.9	> 0.5
Pentobarbital " 50 " S.C.	6	2.60	34.95 $\pm$ 14.49	+ 13.18 $\pm$ 4.31	< 0.01	80.8	+ 29.9	> 0.1, < 0.2
Neocatergan maleate, 2.5 mg/kg I.V.	9	2.33	19.44 $\pm$ 5.99	+ 2.33 $\pm$ 2.80	> 0.4	55.6	— 5.5	> 0.5
Phenergan HCl, 1.5 mg/kg I.V.	7	2.74	26.21 $\pm$ 10.84	+ 4.44 $\pm$ 3.59	> 0.2	57.1	— 4.0	> 0.5
" " 20 " S.C.	8	2.69	24.53 $\pm$ 6.05	+ 2.76 $\pm$ 2.90	> 0.3	12.5	— 48.6	< 0.01
Atropine sulfate, 5 mg/kg I.V.	9	2.37	14.95 $\pm$ 6.31	+ 6.83 $\pm$ 2.44	> 0.02, < 0.05	33.0	— 28.1	> 0.1, < 0.2
SV-25, 0.25 mg/kg I.V.	5	2.40	9.13 $\pm$ 0.66	— 12.64 $\pm$ 3.27	< 0.01	0	— 61.1	> 0.01
SV-25, 2.5 mg/kg I.V.	6	2.36	10.83 $\pm$ 2.31	— 10.94 $\pm$ 3.92	< 0.01	0	— 61.1	< 0.01

\* Statistically significant when 0.05 or less.

† Mortality figures are those based on the numbers of animals dying spontaneously during 15-minute observation period following epinephrine. ‡ Intravenous pretreatment was instituted 10 minutes before epinephrine. Drugs administered subcutaneously were given 30 minutes before the standard intravenous dose of epinephrine of 0.375 mg/kg (as the base).

§ N-(2-bromoethyl)-N-ethyl-1-naphthalenemethylamine · HBr. || N-(2-chloroethyl)-N-ethylbenzylhydrazine · HCl.

<sup>4</sup> Auer, J., and Gates, F. L., *J. Exp. Med.*, 1917, **20**, 201.

<sup>5</sup> Bariety, M., and Kohler, D., *Compt. rend. Soc. de biol.*, 1948, **142**, 280.

<sup>6</sup> Luisada, A., *Arch. f. Exp. Path.*, 1928, **132**, 313.

<sup>7</sup> Boivin, G., *Compt. rend. Soc. de biol.*, 1929, **101**, 251.

<sup>8</sup> Halpern, B. N., Hamburger, J., and Cruehand, S., *Acta allergologica*, 1948, **1**, 97.

<sup>9</sup> Elkeles, A., *Brit. J. Radiol.*, 1948, **21**, 472.

<sup>10</sup> Loew, E. R., and Mietlich, A., *J. Pharm. and Exp. Therap.*, 1948, **93**, 434.

<sup>11</sup> Nickerson, M., and Goodman, L. S., *J. Pharm. and Exp. Therap.*, 1947, **80**, 167.

<sup>12</sup> Rothlin, E., *Bull. de l'Acad. Suisse des Sci. Med.*, 1946-47, **2**, 1.

made by dissolving the base in saline with the addition of a few drops of HCl. Concentrations of other drugs in saline were adjusted so that a given dose was administered in 0.5 ml/kg. Control data were obtained concurrently from animals receiving only epinephrine or no treatment whatsoever. Following the intravenous injection of epinephrine, the rabbits were observed for 15 minutes, at which time, if surviving, they were sacrificed by a sharp blow on the head. All lungs were removed 10 minutes after death, freed of adhering tissue, blotted on absorbent paper and wet-weighted. The indication of effectively altering the toxic response to epinephrine was the presence of a statistically significant reduction in lung weight and/or mortality from that of the controls receiving only epinephrine (Table I).

**Results and discussion.** The lungs of epinephrine-treated rabbits were markedly hemorrhagic and edematous and weighed double that of normal lungs. A frothy fluid, usually uncolored but sometimes salmon colored, frequently completely filled the trachea and bronchi and appeared to account for the respiratory embarrassment, cyanosis and asphyxial convulsions which preceded death. Fluid frequently escaped from the nostrils and mouth during asphyxial convulsions or when survivors were killed 15 minutes after injection of epinephrine. This fulminating, severe pulmonary edema was probably the main cause of death which occurred in 3 to 15 minutes in 61% of the 18 control animals. Pretreatment with a variety of drugs was instituted in attempts to prevent the edema. During each daily experiment several control rabbits and several rabbits treated with each drug were included, thus yielding data which could be pooled and compared (Table I).

**Barbiturates.** Luisada<sup>6</sup> reported that hypnotics in general were capable of reducing the mortality and pulmonary edema induced with epinephrine in rabbits. He believed that such drugs protected by suppressing a central component of a contributory pulmonary vascular reflex which was brought about by epinephrine or the resultant severe hypertension.<sup>6</sup> The results herein reported with pentobarbital in

a near anesthetic dose and phenobarbital in a sedative dose do not support such a contention. Neither of these barbiturates exerted any protective action under conditions which appear to be essentially identical to those described by Luisada. Phenobarbital actually caused a significant increase in lung weight and the data suggest an increased mortality.

**Antihistaminics.** Experiments conducted by Staub<sup>13</sup> indicate that epinephrine may cause the release of significant amounts of histamine in the body. If the toxic effects of large doses of epinephrine in rabbits are accentuated by histamine released from tissues, it would be expected that pretreatment with antihistaminics would confer protection.

The treatment with Pyranisamine (Neonatergan maleate<sup>†</sup>), a potent and specific antihistaminic did not prevent the increase of lung weight or reduce mortality due to epinephrine (Table I). N- $\beta$ -Dimethylaminopropylthiodiphenylamine.HCl (Phenergan, 3277 RP)<sup>†</sup>, administered intravenously likewise afforded no protection, although larger doses injected subcutaneously reduced mortality but did not decrease pulmonary edema. This suggests that death in rabbits due to epinephrine is not attributable solely to pulmonary edema, or that undetected decreases in degree of edema were sufficient to reduce mortality.

These data do not confirm the results reported by Halpern *et al.*<sup>8</sup> who found that the same large doses of Phenergan under essentially the same experimental conditions completely protected rabbits from the toxic effects of epinephrine. Reuse<sup>14</sup> using the same doses of Phenergan in guinea pigs, reported that this drug reduced mortality due to epinephrine, although the protection conferred by Phenergan was not complete. He states that immediately after epinephrine, the treated animals showed signs of respiratory embarrassment. While this suggests the presence of congestion and edema, no routine examination of the lungs was made. However, the lung weights of 3 such animals were somewhat above nor-

<sup>13</sup> Staub, H., *Experientia*, 1946, 2, 29.

<sup>†</sup> Supplied through the courtesy of Merck and Co., Rahway, N. J.

<sup>14</sup> Reuse, J. J., *Compt. rend. Soc. de biol.*, 1948, 142, 638.

mal, even at 2 hours after epinephrine.

*Atropine.* Various authors<sup>4,5</sup> have claimed that atropine protects against epinephrine-induced pulmonary edema. Phenolic ethers of amino alcohols, possessing atropine-like properties as well as antihistamine and epinephrine blocking properties, are said to protect.<sup>5</sup> On the other hand, significant protection by atropine has been denied.<sup>6,15</sup>

Table I shows that a large intravenous injection of atropine sulfate partially prevented to a significant degree the increase of lung weight. Mortality was not significantly decreased probably because of the small number of animals employed. However, doses less than 5.0 mg/kg did not protect, and the dose necessary to confer protection was relatively large considering the potency of the agent. In addition, the protective effect of atropine is slight when compared to that produced by the adrenergic blocking agents.

*Adrenergic blocking drugs.* It has been shown that N-(2-bromoethyl)-N-ethyl-1-naphthalenemethylamine.HBr (SY-28) is a potent adrenergic blocking agent,<sup>16,17</sup> as well as one of the most potent histamine antagonists known.<sup>16</sup> A closely related compound, N-(2-chloroethyl)-N-ethylbenzhydrylamine.HCl (SY-2) is a specific adrenergic blocking agent.<sup>18</sup> Both of these compounds, in comparatively small doses, prevented death and pulmonary edema due to epinephrine in rabbits, as is shown in Table I. It is emphasized that these were the only drugs studied which completely protected the animals against the toxic effects of epinephrine.

Other adrenergic blocking agents are capable of reducing epinephrine-induced pulmonary edema. Rothlin<sup>15</sup> recently reported that ergotamine had such properties. Dibenamine and various other derivatives of  $\beta$ -chloroethylamine, benzyl-imidazoline (Priscol) and yo-

himbine, when given orally, reduced the toxicity of epinephrine injected intraperitoneally in mice.<sup>10</sup> Under these conditions, the dioxanes, 883F and 933F, were ineffective following moderate doses in mice.<sup>10</sup> Presumably the protective action of most adrenergic blocking drugs in mice is due to the prevention of pulmonary edema since increased lung weights were readily demonstrable after epinephrine injection.<sup>19</sup>

We believe the data obtained from rabbits and herein presented and the findings reported for mice,<sup>10</sup> rats<sup>11,12,15</sup> and rabbits,<sup>6</sup> reveal clearly that pulmonary edema and mortality following epinephrine administration are most effectively reduced by vasodilator drugs which are physiological antagonists of epinephrine and by adrenergic blocking drugs which pharmacologically block excitatory actions of epinephrine such as vasoconstriction and the resultant hypertension.

A more direct examination of the acute hypertensive action of epinephrine, and of the pressures within pulmonary vessels and of the effect of drugs thereon would appear to be the most promising approach to elucidation of factors involved in epinephrine-induced pulmonary edema. Hamilton, Woodbury, and Vogt<sup>20</sup> recorded pressure changes in the pulmonary artery and vein of the unanesthetized dog after large, but non-fatal, doses of epinephrine and observed a rise of pressure in both. They concluded that the increased pressures were due to failure of the left ventricle as a result of the high arterial pressure. Furthermore, direct observations of the heart by Auer and Gates,<sup>4</sup> Johnson,<sup>21</sup> and Barach, *et al.*<sup>22</sup> indicate that the left ventricle fails in the rabbit as well. This suggests that a high pulmonary circulatory pressure prevails after toxic doses of epinephrine and could account for the rapid transudation of fluid into the lungs.

*Summary.* 1. Pulmonary edema and mortality induced in rabbits by intravenous injections

<sup>15</sup> Rothlin, E., Report before Eleventh International Congress of Military Medicine and Pharmacy, Basle, June 2-7, 1947.

<sup>16</sup> Loew, E. R., and Mietlich, A., *J. Pharm. and Exp. Therap.*, 1948, **94**, 339.

<sup>17</sup> Stone, C. A., and Loew, E. R., *J. Pharm. and Exp. Therap.*, 1948, **94**, 350.

<sup>18</sup> Loew, E. R., and Mietlich, A., *Fed. Proc.*, 1947, **6**, 351.

<sup>19</sup> Unpublished observations.

<sup>20</sup> Hamilton, W. F., Woodbury, R. A., and Vogt, E., *Am. J. Physiol.*, 1938, **125**, 130.

<sup>21</sup> Johnson, S., *Proc. Soc. Exp. Biol. and Med.*, 1927-28, **25**, 181.

<sup>22</sup> Barach, A. L., Martin, J., and Eckman, M., *Ann. Int. Med.*, 1938, **12**, 754.



of epinephrine were completely prevented by comparatively small doses of adrenergic blocking drugs, *N*-(2-bromoethyl)-*N*-ethyl-1-naphthalenemethylamine.HBr (SY-28) and *N*-(2-chloroethyl) - *N* - ethylbenzhydramine.HCl (SY-2).

2. It would appear unlikely that epinephrine toxicity and the pulmonary edema involved are importantly related to histamine release from tissues since the antihistaminics, Pyranisamine (Neoantergan maleate) and Phenergan (3277 RP), failed to prevent edema al-

though large subcutaneous doses of Phenergan reduced mortality.

3. The barbiturates, pentobarbital sodium and phenobarbital sodium did not decrease mortality or edema.

4. The available evidence indicates that epinephrine toxicity is most readily reduced by vasodilator drugs which represent physiological antagonists, and by adrenergic blocking drugs which are pharmacological antagonists of epinephrine.

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### 17105. Myelination of the Hypothalamus and Its Relation to Thermoregulation in the Hamster.\*

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Correlation of the ability to regulate body temperature in a cold environment with progressive myelination of the hypothalamus has been previously demonstrated in albino rats.<sup>1</sup> The ability of rats to maintain body temperature is acquired at ages ranging between 18 and 30 days. The earlier literature indicating that function of fiber tracts in the central nervous system may be dependent upon myelination of their component nerve fibers was briefly reviewed in a previous report.<sup>1</sup>

In the present report the results of an investigation of myelination in the hypothalamus of the hamster and the development of its ability to regulate against cold are presented. The investigation seemed indicated in view of the fact that hamsters are more immature at birth than rats.

**Methods and materials.** Six litters of Syrian hamsters were used in the present study. Their body temperatures were recorded by means of a Brown electronic continuous recording potentiometer and copper-constantan thermopiles. The thermopiles were inserted into

the colons of the animals to a depth of 4-5 cm. Animals varying from 6-71 days of age were subjected to environmental temperatures of 5-8°C for periods varying from 30 minutes to 20 hours. After exposure the hamsters were anesthetized and perfused with 3½% potassium dichromate followed by Regaud's solution. The brains were then removed, divided in the median sagittal plane and placed in 5% potassium dichromate where they remained for 10 days at a temperature of 37-39°C. After washing, dehydration, embedding, and sectioning at 20 μ they were stained according to the method of Ulett, Dow, and Larsell.<sup>2</sup> Sections were made in the parasagittal plane to permit study of all areas of the hypothalamus (rostral, caudal and middle) in each section. Representative sections from the lateral hypothalami of all members of a given litter were stained en masse and myelin was quantitated by means of a Photovolt electronic photometer as previously described.<sup>1</sup> In only two instances, because of the difficulty in procuring large litters of hamsters, was a litter-mate control sacrificed with the experimental animals.

\* Supported by a grant from the Office of Naval Research.

<sup>1</sup> Buchanan, A. R., and Hill, R. M., *Proc. Soc. Exp. Biol. and Med.*, 1947, 66, 602.

<sup>2</sup> Ulett, G., Dow, R. S., and Larsell, O., *J. Comp. Neur.*, 1944, 80, 1.

*Results.* The first animal from the first litter of hamsters studied was subjected to cold stress at 6 days of age and the other members were successively stressed on the 13th, 18th, 20th, 25th, and 32nd days. The duration of cold stress was 30 minutes. The fall in body temperature of the first animal was approximately  $20^{\circ}\text{C}$ . The older hamsters gradually improved in ability to regulate until the 32nd day when the temperature of the last animal in the litter fell only  $3^{\circ}\text{C}$ . The concentration of myelin in the dorsolateral areas of the caudal hypothalami, as determined photometrically, increased with age and reached its highest level in the 32-day animal. A parallel increase was evident in the myelin of the medullary pyramids but the concentration was considerably higher than in the hypothalamus at all ages.

The members of the second litter were cold-stressed for 30 minutes at ages ranging from 12-36 days. Regulation was best in the 31-day animal in which hypothalamic myelin was also at its highest level. The same was true of Litter No. 3 which was cold-stressed (30 minutes) at ages ranging from 18-46 days. Deterioration in regulatory ability and some apparent decrease in myelin were observed in the 3 members of this litter over 31 days of age (Fig. 1).

Cold stress was continued for 2 hours in the fourth litter studied (Fig. 2). Body temperatures of these hamsters averaged approximately  $1^{\circ}\text{C}$  lower at the end of 2 hours in the cold room than at 30 minutes. Myelin reached its highest level in the 25- and 30-day animals and regulatory ability was best at 30 days. Myelin appeared to decrease in hamsters over 30 days of age and there was an associated deterioration of regulatory ability which was most evident in the 55-day animal after 2 hours of exposure.

There were 10 hamsters in the fifth litter studied which may account for the fact that attainment of regulatory ability was somewhat delayed. Regulation was best at 42 days with myelin content of the caudal hypothalami increasing rather rapidly from 18-42 days. Regulation in the 50-, 60-, and 71-day animals was only slightly less efficient than in the younger animals and was actually better after

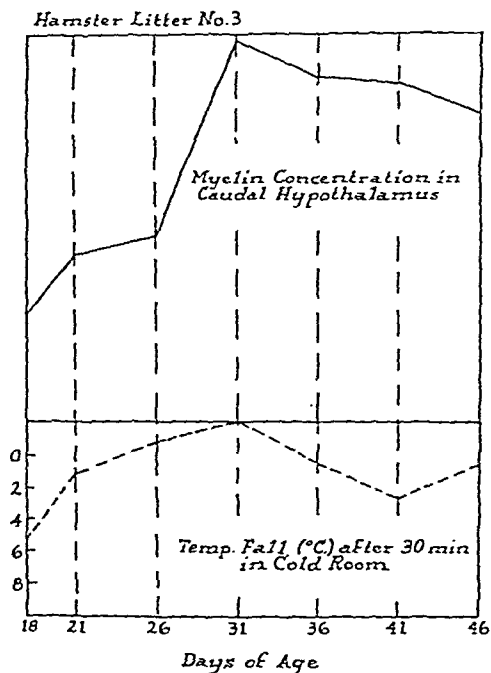


FIG. 1.

Temperature falls after 30 min. at  $5-8^{\circ}\text{C}$  and myelin concentrations in the caudal hypothalami of hamsters in Litter No. 3.

8 hours of exposure to the cold environment than after 2 hours. Myelin concentration in the hypothalami of the 3 oldest members fluctuated between levels slightly above and slightly below that of the 42-day animal.

The sixth and last litter also consisted of 10 hamsters, the first of which (18 days) was not included in Fig. 3 since its temperature, after 2 hours of exposure, had dropped below the range of available recording equipment. Regulation against cold was best at 44 days at which time myelin in the caudal hypothalamus was also at its highest level. Litter-mate controls were sacrificed with the 40- and 59-day stressed animals of this litter and, in both instances, showed higher levels of myelin in their caudal hypothalami than the stressed hamsters (Fig. 3). The 40-, 44-, 59-, and 69-day animals were left in the cold room for 9, 11, 17, and 20 hours respectively; all maintained their body temperatures at levels equal to or higher than those manifested after exposure for 2 hours.

*Discussion.* The experimental data which

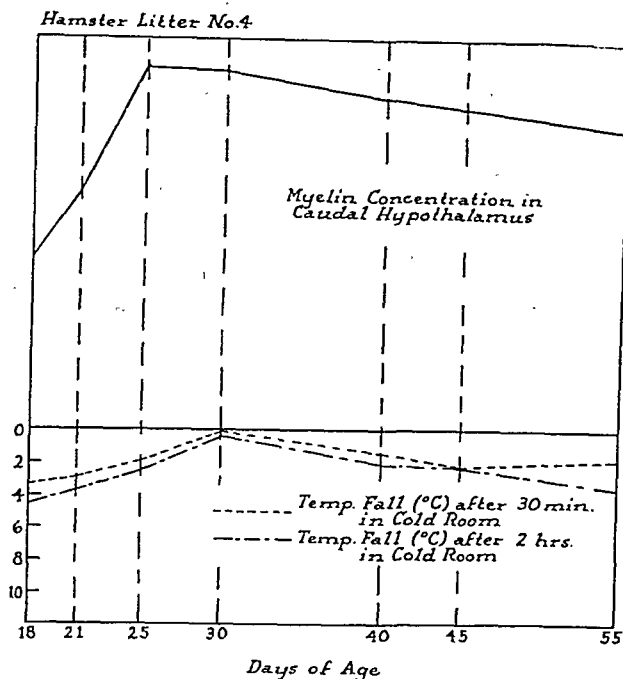


FIG. 2.

Temperature falls after 30 min. and 2 hr in the cold environment and myelin concentrations in the caudal hypothalami of hamsters in Litter No. 4.

have been obtained from subjecting hamsters to an environmental temperature of  $5-8^{\circ}\text{C}$  indicate that their attainment of ability to regulate against cold is considerably more delayed than in albino rats, or 32-44 days of age as compared to 18-30 days. This was to be expected since the gestation period in hamsters is considerably shorter than in rats with the result that hamsters are more immature at birth.

The maintenance of body temperature by reasonably mature hamsters in an environment ranging between  $5^{\circ}$  and  $8^{\circ}\text{C}$  for periods of 20 hours or more is not surprising. Sexually mature hamsters over three months of age were kept in an environmental temperature of  $4^{\circ} \pm 2^{\circ}\text{C}$  for periods of one or two months by Lyman<sup>3</sup> without appreciable reduction from normal of their body temperatures until they entered hibernation. Most of his hamsters "entered hibernation within a month or two after being brought into the cold room";

during hibernation their temperatures were only about  $1^{\circ}\text{C}$  above that of the environment.

As is the case in rats,<sup>1</sup> myelination, determined photometrically, reaches its highest level in the caudal hypothalamus of the hamster at the age when regulatory function is established. This observation, in an additional rodent, supports the hypothesis previously advanced that the role of the hypothalamus in thermoregulation is facilitated, at least in part, by myelination of the axons of hypothalamic neurons.

The observation that myelin concentration in the hypothalamus is higher, according to our method of measurement, in unstressed than in stressed animals from the same litter and at the same age suggests mobilization or change in chemical composition of myelin as a part of the metabolic reaction to cold stress. This may be a more prominent feature in older animals in which the process of primary myelination is essentially complete and it may account for the apparent tendency for myelin

<sup>3</sup> Lyman, C. P., *J. Exp. Zool.*, 1948, 109, 55.

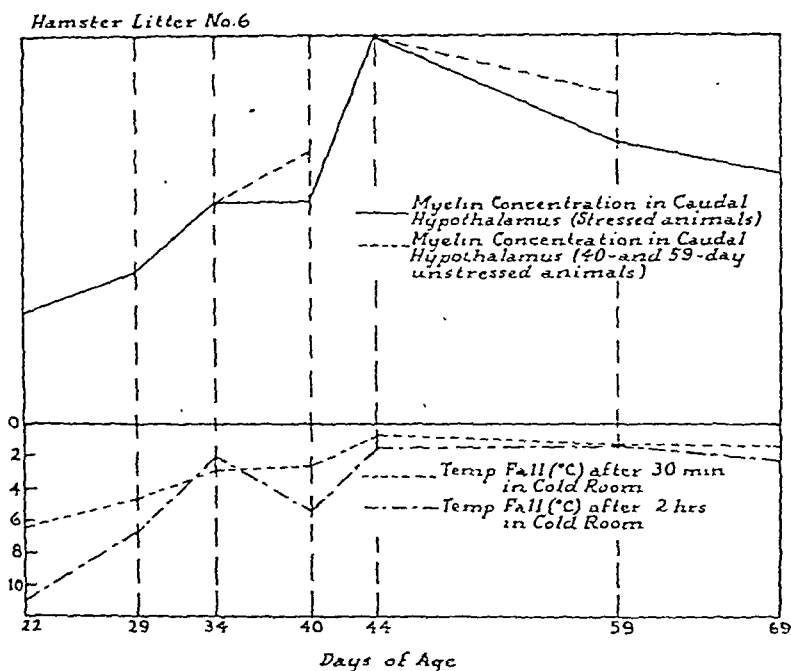


FIG. 3.

Temperature falls after 30 min. and 2 hr in the cold environment and myelin concentrations in the caudal hypothalami of hamsters in Litter No. 6. The differences between myelin concentrations in stressed and unstressed animals at 40 and 59 days of age are also shown.

to decrease after reaching its highest level in hamsters and in the rats previously studied.<sup>1</sup> All the animals heretofore used in plotting myelin curves have been stressed animals, sacrificed immediately after removal from the cold environment. It is unlikely that the mild but consistent deterioration in regulatory ability evident in the older animals (Fig. 2 and 3) is directly related to the decrease in density of stained myelin in the hypothalami, but the possibility cannot be overlooked.

**Summary.** Hamsters acquire the ability to effectively regulate their body temperatures in

a cold environment ( $5^{\circ} - 8^{\circ}\text{C}$ ) at ages ranging from 30-44 days. Myelination in the dorsolateral areas of the caudal hypothalamus, as determined photometrically, reaches its height at the time thermoregulation becomes fully developed. It appears therefore that myelination of hypothalamic neurons may be essential to functional maturation of the hypothalamus. The data with respect to hamsters are similar and lend support to those previously reported for albino rats.

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## 17106. Effect of BAL on Experimental Polyarthritis of Rats.\*

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BAL (2:3-dimercaptopropanol) is used in counteracting gold toxicity which appears occasionally during chrysotherapy of rheumatoid arthritis in man. Experimental polyarthritis of rats produced by the L-4 strain of a pleuropneumonia-like organism likewise responds to gold therapy. Although the experimental disease in rats is pathologically not the same as rheumatoid arthritis in man it has been used as a means of making chemotherapeutic trials.<sup>1</sup>

The use of BAL in arthritic patients who are being treated at the same time with gold raises the possibility that the arthritis may be aggravated because the BAL would increase the excretion of gold and so decrease the amount of gold available for the disease process.<sup>2,3</sup> Since gold has a beneficial action in arthritis the use of a substance capable of facile combining with it might *per se* aggravate the arthritis. To examine this possibility the effect of the administration of BAL on the experimental arthritis of rats, not treated with gold, was studied.

**Procedure.** A total of 41 male and 49 female rats were given a total cumulative dose of 90 to 100 mg per kg body weight of BAL intramuscularly during a period of 6 weeks. For the first 5 days of the experiment 5 mg per kg of BAL (diluted in olive oil) was administered twice daily. Thereafter, the same dose was given twice weekly for 5 weeks.

On the second day of BAL injection the rats received 2.0 cc of a broth culture of the L-4 strain of pleuropneumonia-like organism

intraperitoneally. The organisms were cultured according to a method described by Tripi and Kuzell.<sup>1</sup> Evaluation of the arthritic involvement was made by using a modification of the arthrogram described by Sabin and Warren.<sup>4</sup> This consists of a scoring square divided into 4 quarters. Each quarter represents one leg of the rat. Arthritis is noted by blacking out various portions of the square. A means of scoring these arthrograms according to the degree and extent of rat polyarthritis has been described elsewhere.<sup>5</sup> The arthrogram score is based on a possible score of 4 for a front leg and 5 for a hind leg, giving a maximum score of 18 for the animal.

A total of 37 male and 43 female rats was used as controls and received only the pleuropneumonia-like organism intraperitoneally. Ten rats injected with olive oil alone in comparable dosage showed no difference from these controls. There was no demonstrable change in growth of pleuropneumonia-like organisms in broth cultures *in vitro* to which pure BAL<sup>†</sup> had been added in concentrations of from 0.0007 to 0.2 mg per cc. Infected broth cultures containing BAL in a concentration of 0.2 mg per cc were injected intraperitoneally into rats and the typical arthritis was produced. Increasing the concentration of BAL in the broth cultures beyond 0.2 mg per cc inhibited the growth of the microorganism. Five rats (average weight 60 g) were given 5 mg per kg of BAL intramuscularly twice daily for 5 days and thereafter twice weekly for 5 weeks. These rats were compared with similar untreated controls regarding weight gain. There was no demonstrable dif-

\* This work was done in part under contract with the Office of Naval Research, U. S. Navy Department, and supported, in part, by the Stern Fund for Experimental Arthritis.

<sup>1</sup> Tripi, H. B., and Kuzell, W. C., *Stanford Med. Bull.*, 1947, 5, 98.

<sup>2</sup> Macleod, J. G., *Ann. Rheum. Dis.*, 1947, 7, 143.

<sup>3</sup> Kuzell, W. C., Pillsbury, P. L., and Gellert, S. A., *Stanford Med. Bull.*, 1947, 5, 197.

<sup>4</sup> Sabin, A. B., and Warren, J., *J. Bact.*, 1940, 40, 823.

<sup>5</sup> Tripi, H. B., Gardner, G. M., and Kuzell, W. C., *Proc. Soc. Exp. Biol. and Med.*, 1949, 70, 45.

<sup>†</sup> Supplied by Hynson, Westcott and Dunning, Inc., Baltimore, Md.

TABLE I.

Effect of BAL on Incidence and Extent of Arthritis and on Survival Rate of Infected Animals.

	Arthritis		Survival, %
	Incidence, %	Extent (Arthrograph Avg)	
BAL + pleuropneumonia infection	73	2.86	89
Pleuropneumonia infection only	55	1.83	95

The standard error of difference in incidence of arthritis is 7.3% while the observed difference is 18% so that the chance occurrence of the observed difference is 1:80.

ference in weight between these groups, thus indicating that the BAL had little general toxic effect on the rats.

**Results.** The arthrograph scores given in Table I compare the average arthritic involvement in the group receiving both BAL and the infection with that receiving the infection only. The extent of the average arthritic involvement in the individual animals receiving BAL is greater than in the infected controls. The survival rate is slightly less in the group receiving BAL. The incidence of arthritis is greater in the BAL medicated group.

**Discussion.** These results indicate that the administration of BAL increases the severity of an experimental arthritic process. Such a difference might be attributable to the general toxic effects of BAL rather than to direct enhancement of the growth of the infecting organism, but our failure to show a difference in weight between animals given BAL alone and untreated controls argues against this possibility. Peters *et al.* found that LD<sub>50</sub> from the subcutaneous administration of BAL in oil to rats was 110 mg per kg body weight.<sup>6</sup> Waters and Stock<sup>7</sup> reported the American Reference Standard LD<sub>50</sub> to be 105 mg per kg intramuscularly in rats. It would, therefore, appear that single doses of 5 mg per kg would not be expected to produce appreciable toxicity. The question then arises as to whether the slight toxic effects produced by low doses could increase the severity of the arthritis. This does not seem likely, since it has been found that other toxic substances such as 0.1% sodium phenobarbital, 0.01%

arsenic trioxide, or 0.1% isopropyl alcohol in the drinking water for prolonged periods caused no increase in the severity of this experimental polyarthritis of rats.<sup>8</sup>

It is possible that BAL in low concentration might stimulate growth of the pleuropneumonia-like microorganisms and thus increase the severity of the arthritis. Fildes and Richardson<sup>9,10</sup> have shown that *Staphylococcus aureus* is able to utilize some mercapto compounds as a source of sulphur and, in fact, that sulphydryl compounds are essential metabolites for many cells. Thus, if lack of sulphydryl groups prevents the metabolism of bacterial cells it is possible that an abundance of these SH groups as furnished by BAL might actually stimulate the microorganisms *in vivo*.

**Conclusions.** BAL(2:3-dimercaptopropanol), administered to rats with polyarthritis produced by infection with L-4 strain of pleuropneumonia-like microorganism, causes a significant increase in incidence of arthritis, a more extensive type of joint involvement, and a slightly decreased rate of survival. Concentrations of BAL up to 0.2 mg per cc of broth failed to inhibit or to increase the growth of pleuropneumonia-like organisms *in vitro*. It is suggested that BAL in the low concentrations used *in vivo* may act by furnishing sulphydryl groups needed in the metabolism of the microorganisms, and thus aggravate the polyarthritis, rather than by poisoning the animals and thus reducing their resistance to the infection.

<sup>8</sup> Tripi, H. B., Kuzell, W. C., and Gardner, G. M., *Ann. Rheum. Dis.*, in press, June 1949.

<sup>9</sup> Fildes, P., and Richardson, G. M., *Brit. J. Exp. Path.*, 1937, **18**, 292.

<sup>10</sup> Fildes, P., *Brit. J. Exp. Path.*, 1940, **21**, 67.

<sup>6</sup> Peters, R. A., Stocken, L. A., and Thompson, R. H. S., *Nature*, 1945, **156**, 616.

<sup>7</sup> Waters, L. L., and Stock, C., *Science*, 1945, **102**, 601.

## 17107 P. Pyrogenicity of Influenza Virus in Rabbits.\*

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During an investigation of the toxicity of influenza virus in rabbits, it was noted that the intravenous injection of infected chorio-allantoic fluid was followed by fever. To study this phenomenon, animals were placed in individual stalls, 3 preliminary rectal temperatures were taken at 30-minute intervals to establish a baseline, and the virus preparation to be tested was then injected into the marginal ear-vein. Rectal temperatures were recorded every 30 minutes throughout an observation period of 6 hours after injection. All glassware and saline solutions used were uncontaminated with bacterial pyrogens. A single pool of chorio-allantoic fluid from embryos infected with the PR-8 strain, having a hemagglutinin titer of 1:1024,<sup>1</sup> was used.

One ml of this material consistently caused a rise in temperature beginning 1½ to 2 hours after injection, reaching a peak of 3-4°F above the baseline in the next 4 hours, and gradually falling to normal. Although doses as small as 0.025 ml produced fever, the increases in temperature were of a lower order.

Normal chorio-allantoic fluid and infected fluid from which the virus particles had been removed by centrifugation at 30,000 R.P.M. or by adsorption on chicken erythrocytes gave no fever. The virus resuspended in normal saline solution produced typical temperature elevations.

No fever followed the injection of PR-8 virus neutralized with homologous immune serum.

The febrile response in rabbits is independent of the infectivity of the virus but seems to be related to its adsorptive capacity. Heating at 56°C for 30 minutes destroyed the infectivity of PR-8 for chick embryos but

a portion of the hemagglutinin was retained (1024 to 128); this material produced definite fever in rabbits. Heating at 62°C for 30 minutes destroyed the hemagglutinin titer and these preparations were then non-pyrogenic.

The injection of the Lee strain of influenza B virus produced a fever in rabbits similar to that caused by PR-8. The hemagglutinin of Lee is known to be more heat-resistant than that of PR-8.<sup>2</sup> When the Lee strain was heated at 62°C for 30 minutes it retained adsorptive (1024 to 256) and pyrogenic properties. Exposure to temperatures which destroyed the hemagglutinin rendered the Lee strain non-pyrogenic.

It has been shown that rabbits given a course of daily injections of a bacterial pyrogen become tolerant to the fever-producing effect of that pyrogen and those of other bacterial strains.<sup>3</sup> Animals made tolerant by eight daily injections of 0.1 ml typhoid vaccine responded with typical fevers when given PR-8 on the ninth day, demonstrating that no cross-tolerance existed. The heat stability of bacterial pyrogens, which are not destroyed by boiling,<sup>4</sup> is also in contrast to the lower temperatures at which the fever-producing principle of influenza is destroyed.

Animals which had received 1.0 ml of PR-8 virus on the first day with attendant febrile responses were completely tolerant to the effect of an equal dose on the second day, when no temperature rise occurred. Nine animals given 1.0 ml of PR-8 were re-injected in groups of three on the fourth, seventh, and eleventh days and their temperature responses recorded. As seen in Fig. 1, the responsiveness to the fever-producing effect of the virus gradually returned so that by the eleventh day,

\* The opinions expressed are those of the authors alone and do not necessarily reflect those of the Navy Department or the naval service at large.

<sup>1</sup> Salk, J. E., *J. Immunol.*, 1944, **49**, 87.

<sup>2</sup> Salk, J. E., *Proc. Soc. Exp. Biol. and Med.*, 1946, **63**, 134.

<sup>3</sup> Beeson, P. B., *J. Exp. Med.*, 1947, **86**, 29.

<sup>4</sup> Banks, H. M., *Am. J. Clin. Path.*, 1934, **4**, 260.

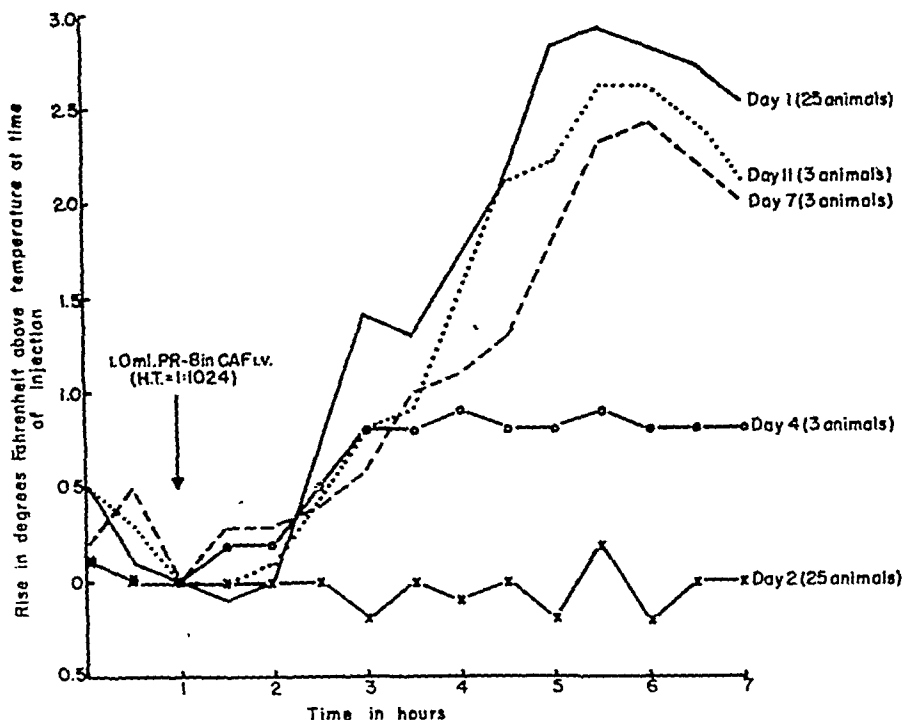


FIG. 1.

Average temperature responses of groups of rabbits to PR-8 influenza virus on day 1 and to a single injection repeated at intervals after the initial injection. Note the complete unresponsiveness on day 2 and the increase in fever through day 11.

their fevers approached first-day levels. Antibody titers (as measured by hemagglutination inhibition) of these animals showed increasing levels during this period when tolerance was being lost. Thus, there is evidence that the resistance to the pyrogenic effect of the virus is unrelated to specific antibody production, although as pointed out above, virus neutralized with immune serum before injection causes no fever.

The observation of Harris and Henle<sup>5</sup> that

an injection of influenza virus will cause lymphopenia in rabbits was confirmed, but this effect on the lymphocytes was not seen on the second day. By the eleventh day, the lymphopenia, like the febrile response, again approached that following the first injection.

<sup>5</sup> Harris, S., and Henle, W., *J. Immunol.*, 1948, 58, 9.

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## 17108. Biologic Absorption of Insolubilized Gelatin Films.

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Our experience in preparing absorbable hemostatic sponges from gelatin<sup>1,2</sup> encouraged us to investigate the possibility of developing a gelatin film that might also be clinically useful. During the course of our studies Bing<sup>3</sup> reported his observations using gelatin sponge at the end of which paper he mentioned without elaboration an incomplete study involving a gelatin film preparation.

The purpose of this report is to submit the data we have obtained from studying the *in vitro* water solubility and enzymatic digestion and *in vivo* biologic absorption of uniquely prepared gelatin films.

Several materials, both absorbable and nonabsorbable, have been employed in surgery as films. Brown, *et al.*<sup>4</sup> in their report on the use of polythene film mentioned most of these and noted the limitations of each. Oppenheimer's<sup>5</sup> report suggests that nonabsorbable substances must be observed for a sufficient period to assure that they are not carcinogenic since these investigators noted that cellophane implants induced sarcomas in rats after remaining *in situ* more than 11 months.

**Materials and Methods.** Films were prepared from commercial pigskin gelatin. To remove cations each 100 g of raw, ground gelatin was eluted for 2 hours with one liter of cold (5°C) M/128 glacial acetic acid; the acid was drawn off and the gelatin mass washed several times with cold double distilled water.

Usually 5% solutions were prepared from this washed gelatin. To these were either

added nothing or various small quantities of U.S.P. formalin. The mixture was incubated at 37°C for 2 hours after which films were cast, dried on stainless steel plates under controlled humidity and heated at 140°C for different lengths of time. The investigations which are reported here were carried out on films that were approximately .003 inch thick. Films of such light weight, even when dry, are remarkably pliable and only break when folded sharply.

To obtain sterile preparations, when desired, the gelatin films were subjected to routine dry heat (140°C for 4 hours) sterilization in accordance with the official U.S.P. method.<sup>6</sup>

Three criteria were employed to establish to what degree a given treatment had altered the gelatin molecule yielding films with varying physical and biologic characteristics.

The first of these was the tendency of the film to go into water solution. To uniform quantities of film was added an appropriate volume of distilled water which stood on the gelatin at room temperature overnight. The next morning the solution was heated on a steam bath with occasional stirring and the films observed until dissolved or for at least 24 hours.

In another test the time for *in vitro* digestion<sup>1</sup> of films by pepsin was determined. To pieces weighing 150 mg was added 100 cc of a 1% U.S.P. pepsin solution. The containers were placed in a water bath held at 37°C and mechanically agitated; the time was noted when the last fragment had been digested.

Finally biologic absorption and possible reaction were investigated by implanting pieces that weighed roughly 15 mg (ca. 10 x 12 mm) usually in muscle layers, in the legs of rats using a technique similar to one that has been previously<sup>2</sup> described. Sixteen albino rats weighing around 300 g were prepared in each series.

<sup>1</sup> Correll, J. T., and Wise, E. C., *Proc. Soc. Exp. Biol. and Med.*, 1945, **58**, 233.

<sup>2</sup> Correll, J. T., Prentice, H. R., and Wise, E. C., *Surg. Gynec. and Obst.*, 1945, **81**, 585.

<sup>3</sup> Bing, J., *Acta Pharmacol. and Toxicol.*, 1947, **3**, 364.

<sup>4</sup> Brown, M. H., Grindlay, J. H., and Craig, W. McK., *Surg. Gynec. and Obst.*, 1948, **86**, 663.

<sup>5</sup> Oppenheimer, B. S., Oppenheimer, E. T., and Stout, A. P., *Proc. Soc. Exp. Biol. and Med.*, 1947, **67**, 33.

<sup>6</sup> *Pharmacopoeia of the United States*, thirteenth revision, Mack Publishing Co., Easton, 1947.

TABLE I.  
*In vitro* and *in vivo* Evaluations of Absorbable Gelatin Films.

Film preparation		Response in hot water	Proteolytic digestion time	Biologic absorption
No.	Treatment			
B-98-1	Untreated	In solution in 1 to 2 min.	Film soluble	Gone in less than 24 hrs.
B-98-2	Heated 2 hrs	In solution in 7 to 8 hrs.	70 to 80 min.	
B-98-3	Heated 4 hrs	Disintegrated into fragments in 24 hrs.	1½ to 2 hrs.	Gelatinous in 1 day; gone in 4 days
B-93-1	.03% formalin; not heated	In solution in 30 min.	40 min.	
B-93-2	.03% formalin; heated 4 hrs	Much swelling; no solution in 24 hrs.	2 to 4 hrs.	
B-93-3	.03% formalin; heated 8 hrs	Some swelling; no solution in 24 hrs.	5 to 8 hrs.	Gelatinous in 2 weeks; gone in 7 to 8 weeks
B-97-1	.06% formalin; not heated	In solution in 25 min.	60 to 80 min.	
B-97-2	.06% formalin; heated 4 hrs	Some swelling; no solution in 24 hrs.	5 to 8 hrs.	Gelatinous in 2 weeks; gone in 7 to 8 weeks

**Results.** The data obtained from several different investigations have been organized into a single table (Table I) for presentation. Values are reported in ranges to indicate that the tests cannot be reproduced absolutely from one experiment to the next, but that they do indicate direction. Thus it can be predicted that a film which will finally go into solution in water in 7 to 8 hours (B-98-2) will be digested by pepsin more rapidly than a film which only swells and will not dissolve in water (B-93-2).

It is our experience also that insolubilized films with short enzymatic digestion times (B-98-1, B-98-3) will soften and be resorbed in the body at a faster rate than those with appreciably longer (B-93-3, B-97-2) *in vitro* times.

Enzymatic digestion times and biologic absorption do not, however, have a straight line relationship. For we have observed with gelatin films, as Morrison and Singer have previously noted with fibrin films,<sup>7</sup> that variations in the mode of preparation may result in greatly prolonged *in vitro* digestion times with inconsiderable if any variation in the observed *in vivo* resorption. In experiments not recorded in the table we have prepared insolubilized gelatin preparations which remained undigested by pepsin after 24 hours yet were

absorbed biologically in periods of time only slightly longer than films which were insolubilized to a lesser degree according to their *in vitro* characteristics.

Resistance to water solubility was imparted to the film by hot air heating of the dried film (B-98-2, B-98-3) or by the addition of minute quantities of formalin (B-93-1, B-97-1) to the original solution. A combination of the two techniques resulted in a greater degree of insolubilizing (B-93-2, B-93-3, B-97-2). From the table it can be seen that, generally, the more intensive the treatment by either or both of the methods the more altered the film according to the tests that were applied.

Biologic implants of films that had *in vitro* proteolytic digestion times of less than 4 hours softened in the muscle tissue rapidly and no residue could be detected upon gross inspection by the fourth day (B-98-3).

Because it seemed likely that such films would be clinically impractical, preparations were made that were more resistant to *in vitro* digestion. *In vivo* these were found to withstand undue disintegration for 8 to 10 days, to be gelatinous in about 2 weeks and be absorbed in 7 to 8 weeks (B-93-3, B-97-2).

In the series in which gelatin film B-98-3 was implanted in the left thigh muscles a comparable piece of regenerated cellulose (Du Pont cellophane No. 600 PT) was implanted in the same area in the right side. On inspec-

<sup>7</sup> Morrison, P. R., and Singer, M., *J. Clin. Invest.*, 1947, 26, 929.

tion as long as 11 weeks after implantation the cellophane was readily located encapsulated in a thin membranous envelope; it showed no evidence of disintegration or resorption. Lowry<sup>8</sup> reported a tendency of certain cellophane films which he studied to be absorbed. In the studies of Oppenheimer *et al.*<sup>9</sup> the regenerated cellulose which they implanted was unabsorbed over a period of 11 months or more.<sup>9</sup>

In addition to the gross observations which are recorded here, specimens of the implant areas were fixed and submitted for histologic examination; the results of the histologic readings will be reported in detail in another paper. Since the properties of water insoluble gelatin film suggested that it might have clinical utility wherever an absorbable membrane is indicated, this possibility is being independently investigated in several surgical research laboratories.

<sup>8</sup> Lowry, M. L., *Arch. Surg.*, 1946, **52**, 160.

<sup>9</sup> Oppenheimer, B. S., personal communication, November 1948.

**Summary.** The preparation and properties of gelatin films, which resisted water solution in varying degrees, have been described.

These films, although water insoluble, have been shown to be digested in different lengths of time when subjected to proteolytic enzymes *in vitro* in a standardized assay.

Some of the altered gelatin films were implanted into muscle areas of albino rats. Upon visual inspection they were found to be absorbed at the recorded intervals, with no evidence of untoward tissue reaction. The data indicate that the biologic absorption time can be roughly predicted from *in vitro* digestion results; as the *in vitro* times become greatly prolonged, however, a comparable resistance to biologic absorption is not seen.

Cellophane implants were not absorbed but at the eleventh week were encapsulated in a thin membranous capsule.

The authors are grateful to Margaret Borst for her assistance in this work.

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### 17109. Sodium Acetate as a Source of Fixed Base.\*

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Recent studies have shown that acetic acid is an extremely rapidly oxidized product of the intermediary metabolism of both carbohydrate and fat.<sup>1</sup> From the large turnover of acetate in the normal metabolic processes of the body, it is obvious that sodium acetate should serve as a rapidly available source of fixed base. Using the change of serum CO<sub>2</sub> content as an index of the presumptive rate of oxidation of the acetate ion, studies were performed in dogs and in man to define the usefulness of sodium acetate as a source of fixed base.

**Procedure and results.** Dogs, anesthetized with pentobarbital, were infused intravenously with a solution of 1/6 molar sodium acetate, the total amount administered being 15 mEq per kg of body weight. Blood specimens were taken under oil at intervals of 15 minutes or longer, and serum CO<sub>2</sub> was determined by the manometric method of van Slyke. Studies on 8 dogs yielded uniform results and showed a rapid rise in CO<sub>2</sub> content. The observations from 5 experiments with similar rates of infusion are given in Table I. Within 15 minutes after starting the sodium acetate infusion the Serum CO<sub>2</sub> showed a prompt elevation and continued to rise during the infusion, leveling off in the afterperiod without further significant change. Infusion of the same

\* Aided in part by a grant from the United States Public Health Service.

<sup>†</sup> National Research Council Fellow.

<sup>1</sup> Bloch, K., *Physiol. Rev.*, 1947, **27**, 574.

TABLE I.  
Effect of Sodium Acetate Infusion on Serum CO<sub>2</sub> Content in the Dog.

Dog	A	B	C	D	E	Avg
Duration of infusion (min.)	75	75	55	65	60	66
Time after starting infusion (min.)	Serum CO <sub>2</sub> volumes %					
0	51.5	51.0	53.7	56.2	52.4	53.0
15	60.4	62.4	64.8	68.7	64.1	64.1
30	74.1	75.0	82.0	80.2	75.6	79.4
60	88.8	89.3	92.6	99.8	99.2	93.9
120	97.0	97.1	100.2	105.8	102.2	100.5
180	97.0	98.1	104.1	107.4	102.1	101.7
240	—	98.8	104.0	106.8	102.0	102.9

quantity of sodium acetate twice as quickly promoted a rise which was less marked, thus suggesting that the renal threshold for acetate had been exceeded. Because of the reported vasodilator action of acetate,<sup>2</sup> blood pressures and electrocardiograms were taken, but no changes or abnormalities were noted with the doses employed.

Solutions of 1/6 molar sodium acetate were prepared and autoclaved in the routine manner for use in patients. Normal adult subjects and patients with renal acidosis were studied in the fasting state. Infusions of 1 liter were given within a one hour period. The average dosage, uncorrected for differences in weight of patients, was 2.8 mEq per kg of body weight, approximately one-fifth of the amount given per kg to the dogs. No attempt was made to define the upper limit of tolerance in human subjects. The amounts used satisfy reasonable clinical requirements. Blood pressures and pulse rates were determined at frequent intervals and no significant changes were noted, nor were any subjective symptoms or other evidence of toxicity observed. The results of the serum analyses are shown in Fig. 1. The rapid rise of serum CO<sub>2</sub> was evident within 15 minutes and the rise continued progressively. Except in 2 subjects, there was no further increase in serum CO<sub>2</sub> after termination of the infusion. The data have been presented without correction for body weight, body water or renal excretion, and although small variations could be accounted for by these factors, it is evident that acetate is rapidly metabolized with the re-

lease of fixed base. Three subjects were given one liter of 1/6 molar sodium bicarbonate in one hour as a control study. As seen in Fig. 1, the rate of increase in serum CO<sub>2</sub> content was essentially the same for the sodium bicarbonate infusion as it was for the sodium acetate, indicating, within the limits of the methodology employed, that there was an almost instantaneous utilization of the acetate. In 3 patients the urine became sharply alkaline fol-

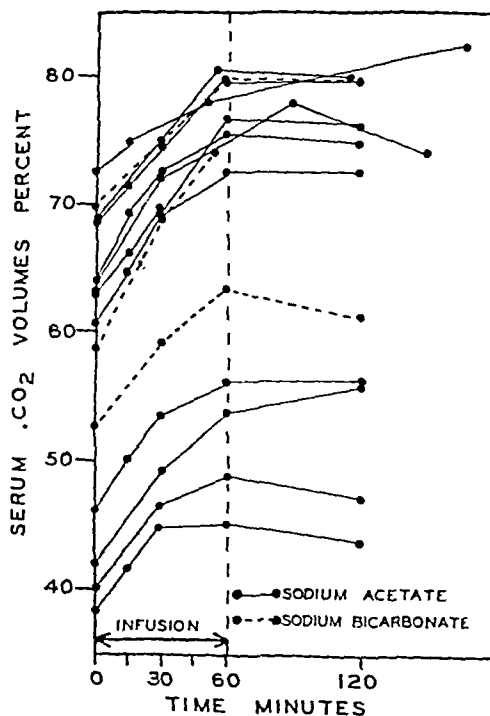


Fig. 1.

Comparison of effect of sodium acetate and sodium bicarbonate infusions on serum CO<sub>2</sub> content in man.

<sup>2</sup> Bauer, W., and Richards, D. W., Jr., *J. Physiol.*, 1928, 60, 371.

lowing the infusion of the sodium acetate.

**Discussion.** Sodium acetate affords a readily procurable and inexpensive source of fixed base, as rapidly and as completely available as sodium bicarbonate. Solutions are easily prepared, easily sterilized, and indefinitely stable. The pH of 5 different lots of sodium acetate made up in 1/6 molar concentration was found to vary from 7.6 to 8.0. We believe that the use of sodium acetate solutions is indicated in rapid parenteral alkalization and should be useful in the therapy of most types of metabolic acidosis. However, we wish to emphasize that because the metabolism of acetate has not been specifically defined as to its possible effect on ketone body production in diabetic acidosis, we have not used it to date in this condition.

These studies have not revealed previously

undescribed properties of sodium acetate. Indeed the salt has been recommended in standard textbooks<sup>3,4</sup> as a source of fixed base, but its clinical use has been limited. The present observations demonstrate the rapid catabolism of acetate and emphasize certain practical advantages which it possesses for clinical use.

**Summary.** Studies in dogs and in man demonstrate that sodium acetate is an easily prepared and a rapidly available non-toxic source of fixed base suitable for parenteral administration when alkalization is indicated.

<sup>3</sup> Goodman, L., and Gilman, A., *The Pharmacological Basis of Therapeutics*, The Macmillan Company, New York, 1941.

<sup>4</sup> Sollmann, T., *A Manual of Pharmacology*, W. B. Saunders Company, Philadelphia, 1948.

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## 17110. A Method for Assay of Serum Proteolytic Activity.\*

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That blood contains an inactive precursor (plasminogen) which can be activated to form a proteolytic enzyme (plasmin) has been established.<sup>1-7</sup> Study of variations in this enzyme have largely been on the basis of its fibrinolytic properties. Using a method of

preparation outlined by Milstone,<sup>6</sup> we have been able to obtain from serum a plasminogen-containing fraction with consistent and reproducible activation in humans and dogs.

**Reagents and Materials.** 1. *Streptokinase*—the filtrate of a 24-hour culture of Group A, Type 11, Beta hemolytic streptococci, grown in beef heart infusion broth with 0.05% added dextrose.

2. *Protamine Sulphate*<sup>§</sup>—a solution of protamine sulphate in veronal buffer, 2 mg per ml.

3. *Veronal Buffer*—pH 7.35<sup>§</sup>

4. *Acetic acid—distilled water mixture*—19 volumes of distilled water plus 0.32 volumes of 1% acetic acid.

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6 Milstone, H. J., *J. Immunol.*, 1941, 42, 109.

7 Ferguson, J. H., Travis, B. L., and Gerheim, O. B., *Proc. Soc. Exp. Biol. and Med.*, 1947, 64, 285.

§ Eli Lilly and Company, Indianapolis, Ind.

8 Clark, D. G. C., Clifton, E. E., and Newton, B. L., *Proc. Soc. Exp. Biol. and Med.*, 1948, 69, 276.

TABLE I.

Sample Reading of Results in %T and Tyrosine Produced After 2, 6, and 24 Hours Incubation with Streptokinase (S), Protamine (P), and Spontaneously (O) Activated Plasminogen. Controlled by Reagent Blanks with 0.5 ml Veronal Buffer Substituted for Plasminogen.

Patient	Activator	% T			Tyrosine—mg		
		2 hr	6 hr	24 hr	2 hr	6 hr	24 hr
B.U.	S	79.5	45	6	.148	.256	.905
	P	91.8	100	59.6	.026	0	.166
	O	93	93.8	84.7	.023	.019	.053
H.D.	S	85	47	7.5	.050	.242	.835
	P	100	90	72	0	.033	.105
	O	96.4	88.5	81	.01	.039	.066
J.S.	S	90.8	86.5	61.5	.03	.045	.155
	P	94	90	58	.018	.033	.175
	O	95	93.5	65	.015	.02	.138
F.G.	S	58.2	24	4.5	.174	.458	1.0
	P	82	69	46.5	.063	.118	.245
	O	80.2	74	58.5	.07	.095	.173
H.C.	S	89	59	18.3	.036	.17	.545
	P	91.2	87.5	69.5	.028	.043	.116
	O	91.3	89.5	86	.027	.035	.048
Reagent blanks	S			100			0
	P			97			.008
	O			100			0

5. *Trichloracetic Acid* (USP), 16%

6. *NaOH* (Reagent grade), 1N

7. *Casein*—a 5% solution, adjusted to a final pH of 7.35-7.45.

8. *Folin-Ciocalteu phenol reagent*—diluted 1:3 with distilled water.

9. *Serum*—obtained from nonhemolyzed blood drawn under sterile conditions.

**Method.** Plasminogen is prepared by adding 1.5 ml serum to 28.5 ml acetic acid-distilled water mixture, inverting once to mix, and centrifuging at 2000 RPM for 15 minutes. The supernatant is discarded and the precipitate dissolved in 1.5 ml veronal buffer; 0.5 ml of this solution, containing plasminogen, is then added to each of three test tubes. To the first, 0.4 ml streptokinase and 0.1 ml buffer are added; to the second 0.5 ml protamine sulphate solution; and to the third, 0.5 ml buffer. 5 ml of casein solution are added to each tube, mixed by inversion, and a 2.5 ml aliquot taken immediately.

5 ml trichloroacetic acid are added to each aliquot, and the resulting coagulum is separated from the sides of the tubes by thorough shaking. Each sample is filtered through

Whatman No. 3 filter paper, and a 2.5 ml aliquot of the filtrate is added to 5 ml of 1N NaOH in a 50 ml Erlenmeyer flask. 1.5 ml of diluted phenol reagent is added to each flask in turn as rapidly as possible, and the contents of the flasks are transferred to spectrophotometric cuvettes. Exactly ten minutes after phenol reagent is added to the first flask, the %

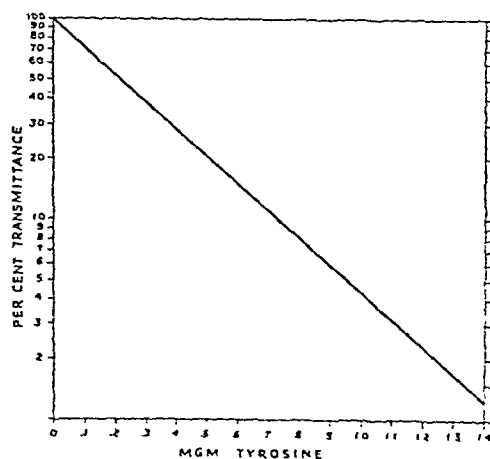


FIG. 1

Calibration curve. Standard tyrosine solution in casein.

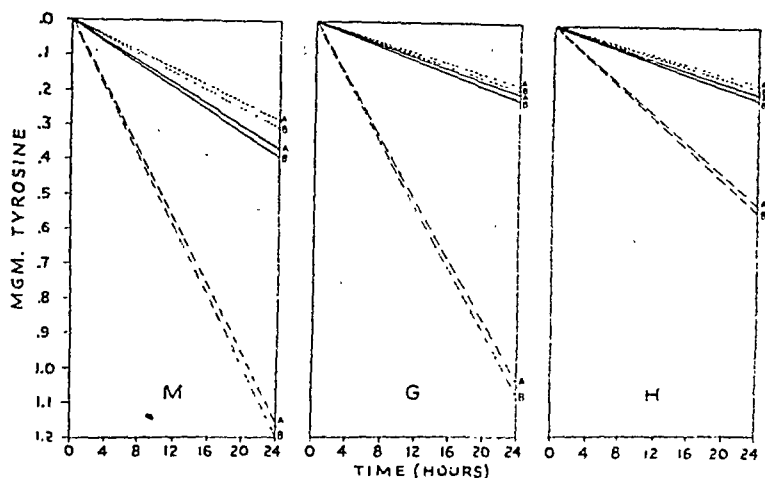


FIG. 2

Graphs of results with duplicate samples of blood of 3 pts. M, G, and H. A and B represent aliquots of each serum taken simultaneously and treated as individual sera.

----- Spontaneous activity.  
 ————— Protamine activated.  
 - - - - - Streptokinase activated.

transmittance of each sample is determined in turn in the Coleman Spectrophotometer, set at 675 mu. The original tubes are incubated for 24 hours at 37°C.

The second 2.5 ml aliquot is taken after 24 hours incubation at 37°C, and color is developed as described above. When the samples are read, the first aliquot acts as the "blank" for the second. A stock solution of a blue dye, T-1824, is prepared so that its optical density is greater than the optical densities of the first aliquots of the unknowns. In reading the first aliquots in the spectrophotometer, the color-producing agents already present in the digest mixtures are negated by setting the galvanometer index at 100% T, or 0 concentration. With this as a reference point, the %T of the dye is then taken for each sample. When the corresponding 24-hour sample is read, the dye, as the "blank", is set at the T value obtained the day before, and the % T of the unknown is then read directly.

**Calculation.** Standard amounts of tyrosine were added to 5 ml of casein solution, and 2.5 ml aliquots were taken and treated as above. These were read against a similar aliquot of casein without added tyrosine. A calibration curve was prepared, so the % T of unknown samples can be expressed directly as mg of

tyrosine produced (Fig. 1, Table 1).

**Results and Discussion.** Aliquots of sera tested after 2, 6 and 24 hours' incubation showed steadily increasing amounts of tyrosine produced, and for experimental purposes the 24 hour period was chosen (Table I). Reproducibility was established by taking aliquots of serum simultaneously and treating them as individual samples (Fig. 2).

Demonstration of the activation of plasminogen by streptokinase and the slow spontan-

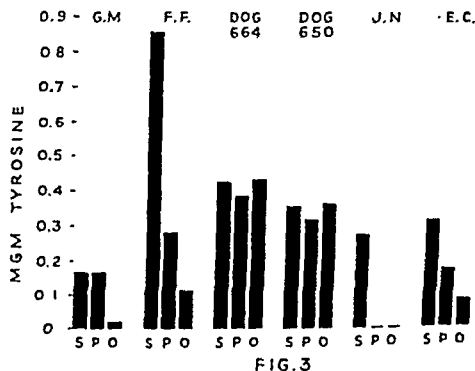


FIG. 3

Columnar graphs showing activation by streptokinase (S), protamine (P), and spontaneous activation (O). 4 representative patients and 2 dogs. Note similarity of activation by agents in dogs as compared to humans.

eous activation confirms previous studies of this relationship. The activation by protamine is definite, though in most instances not as great as is that by streptokinase (Fig. 3). This is at variance with the previous impression that the fibrinolytic activity of protamine was due to inactivation of antifibrinoly-sin.<sup>10</sup> In the present study protamine sulphate (up to 1.2 mg/tube) failed to diminish antiproteolytic activity tested by the method previously reported.<sup>8</sup>

<sup>10</sup> Scroggie, A. E., Jaques, L. B., Rocha e Silva, M., *Proc. Soc. Exp. Biol. and Med.*, 1947, 66, 326.

Dog sera differ from human in that their spontaneous activation is of the same order as the protamine and streptokinase activation (Fig. 3).

*Summary.* A method for preparation of plasminogen from small quantities of blood has been described. Activation by streptokinase, protamine sulphate, and spontaneous activity have been observed. Studies of other activators and of species differences are in progress.

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### 17111. Automatic Measurement of Alveolar CO<sub>2</sub> in Small Animals.

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(Introduced by Albert W. Hetherington.)

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The following paper will describe the application of an infra-red analyzer to alveolar carbon dioxide studies in small animals such as the rabbit, where rapid respiration and small respiratory volumes pose severe problems. As reported elsewhere,<sup>1</sup> this analyzer has been used successfully in measuring the alveolar CO<sub>2</sub> tension in humans during hyperventilation as it is performed in routine electroencephalography. The analyzer was developed for other purposes by Luft<sup>2</sup> and applied to CO<sub>2</sub> measurement by Schmeiser.<sup>3</sup> It measures the infrared absorption of one gas in a mixture by using a fixed quantity of the same kind of gas as a thermomechanical metering device. This method allows an analysis which is rapid enough to fully record a change in CO<sub>2</sub> tension in one-seventh of a second. Thus, in human studies, alveolar air sampling devices are unnecessary, and a portion of the expired air is drawn continuously through the analyzer. The latency of measurement then depends solely upon the speed of transport of

this air through the test chamber. The only additional equipment required is a long expiration tube to prevent dilution of the continuous sample by outside air. The measured CO<sub>2</sub> value after a certain expired volume can be safely regarded as the conventional "alveolar CO<sub>2</sub> tension."

Fig. 1 illustrates the CO<sub>2</sub> analyzer in simplified form. A hot-wire infra-red source (S) projects a beam of infra-red through a transparent (mica) test cell (T) containing the air for CO<sub>2</sub> analysis. The remaining infra-red is absorbed in a following chamber (A), one wall of which is a thin metal membrane. The temperature increase due to the absorption displaces the membrane by increasing the pressure, thus measuring the amount of infra-red reaching the chamber. If the chamber is filled with CO<sub>2</sub>, it becomes sensitive to only those wave lengths which the CO<sub>2</sub> in the test cell absorbs. Thus, it is insensitive to any other gases appearing in the test cell. To balance the system, an identical arrangement (S', C, A') is attached to the other side of the membrane, and the two sides "buck" each other. Displacements of the membrane are measured by utilizing it as one plate of a condenser and

<sup>1</sup> Blinn, K. A., and Noell, W. K., *EEG Clin. Neurophysiol.*, 1949, in press.

<sup>2</sup> Luft, K., *Z. f. Tech. Phys.*, 1943, 24, 97.

<sup>3</sup> Schmeiser, K., unpublished work.



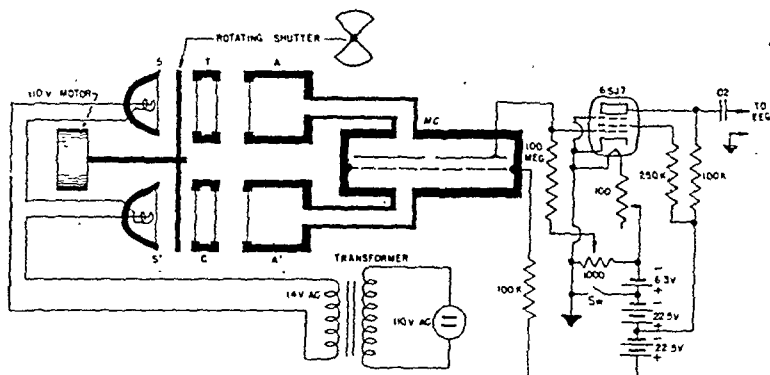


FIG. 1.

Diagram of the CO<sub>2</sub> analyzer and its associated preamplifier. S,S'—hot wire infra-red sources; T,C—test and control chambers; A,A'—absorption chambers of membrane-condenser, MC.

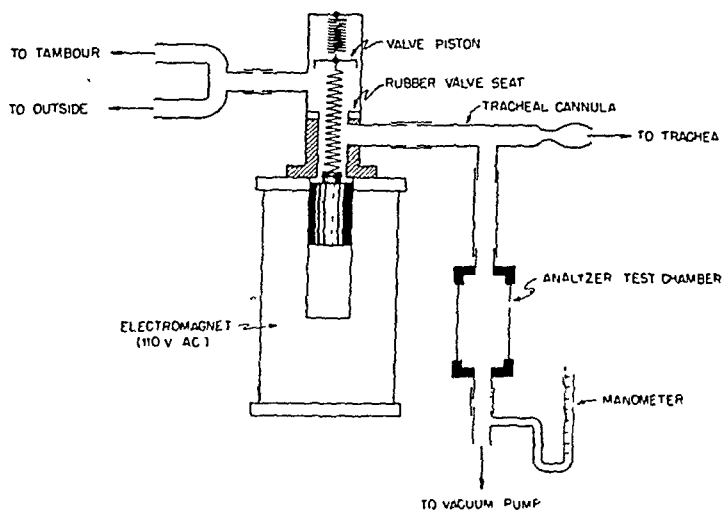


FIG. 2.

The alveolar gas sampling apparatus. The connections between the electromagnetic valve, the tracheal cannula, and the analyzer test chamber are made as short as possible.

measuring changes in electrical charge on the condenser. To produce an alternating voltage for amplification, the two infra-red beams are simultaneously interrupted 7 times per second by a rotating shutter. After amplification the output of the analyzer can be recorded on any instrument, for example, an electroencephalograph. The output appears as a train of 7c/s waves, the amplitude of which is proportional to the CO<sub>2</sub> concentration within limits determined by the thickness of the test cell.

Respiration in humans, even during hyperventilation, is conveniently slow; but in small

animals, such as the rabbit, it may be so rapid that the seven-per-second shutter frequency is proportionally too low, and the respiratory volume exchange becomes a critical factor. Unfortunately, the shutter frequency can be increased only at the expense of a major part of the sensitivity. In these animals, the respiratory rate may exceed 200/minute. Therefore, in order to obtain an undiluted alveolar sample in the test chamber, it was necessary to artificially lengthen a single expiration whenever such a rate prevailed. Fig. 2 shows how this was managed. A tracheal cannula

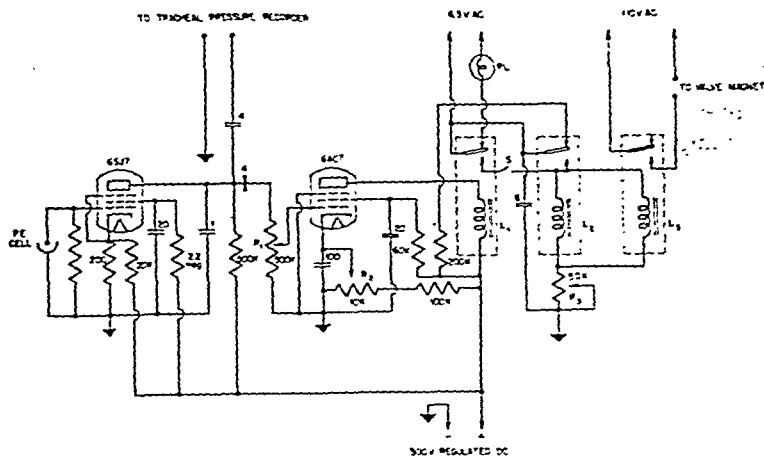


FIG. 3.

The electronic circuit for activating the valve. Bias is adjusted beyond cut-off with  $R_2$ ;  $R_1$  is then advanced until the pilot light PL begins to extinguish at the peak of expiration. When the switch S is closed, the valve magnet is activated for a single sampling of predetermined duration.  $R_3$  varies the sampling duration from  $\frac{1}{2}$  sec. to 2 sec.  $L_1$ ,  $L_2$ , and  $L_3$  are 10,000 ohm relays.

was used, with a side arm leading to the test chamber and suction pump. To obviate disturbances of the respiratory rhythm and vagus effects, an automatic system was devised for briefly closing the cannula at the peak of the expiratory pressure curve. An electromagnetic valve closes the distal end of the tracheal cannula so that an alveolar sample is drawn through the analyzing chamber by the pump. The onset and duration of the closing are controlled electronically in the following manner: tracheal pressure fluctuations are transformed into voltage fluctuations by means of a tambour-operated shutter in front of a photoelectric cell. The electrical fluctuations operate a series of relays (Fig. 3) in such a manner that when a sampling switch is closed, the electromagnetic valve closes at the peak of the next expiration and remains closed only for a preset length of time.

Fig. 4 illustrates the result. The  $\text{CO}_2$  is recorded on the electroencephalograph tape simultaneously with cortical potentials, the EKG, and the tracheal pressure. Each strip is about 12 seconds long. In the top example, the respiration rate is about 180/minute, and the short groups of waves represent the  $\text{CO}_2$  concentrations over single expirations. The highest amplitude varies from one group to another because of the varying

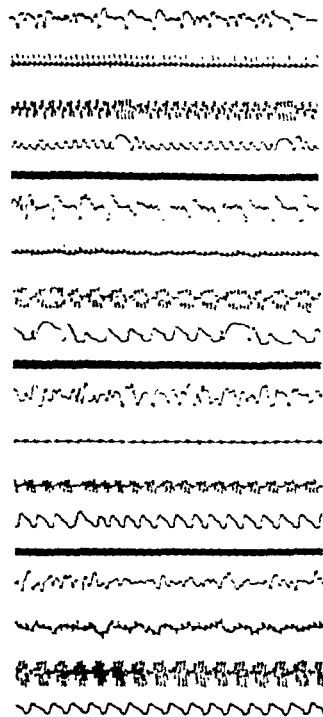


FIG. 4.

Ink-writer recordings illustrating alveolar  $\text{CO}_2$  measurements in a rabbit. From top to bottom, the tracings in each strip are: electrocorticogram (area striata), EKG,  $\text{CO}_2$  tension, and tracheal pressure. Description in the text. The cortical activity is being driven by one-per-second photic stimulation.

amounts of alveolar air caught in the test chamber. The 3 long groups are samples taken automatically. It can be seen that such sampling was necessary at this respiration rate. During the sampling, the heart rate and electrocorticogram do not change, indicating that there were no appreciable disturbances by vagus reflexes. The next example shows a slower respiration of about 60/minute, and here it is obvious that the sampling was unnecessary. The CO<sub>2</sub> concentration does not become any higher during prolongation of expiration. The third example demonstrates conditions under severe hypoxia, which was induced shortly after example 2. The alveolar CO<sub>2</sub> has dropped considerably, and it remains low throughout the gasping period which follows until oxygen is resupplied. The immediate increase to a very high CO<sub>2</sub> concentration is shown in the last strip, which was recorded during post-anoxic convulsions. Under the conditions of the last two examples, automatic sampling was

proven unnecessary and even undesirable because of increased vagus excitability. When such a high excitability is combined with a high respiration rate, the described sampling method is not feasible.

*Summary.* An infra-red CO<sub>2</sub> analyzer having a measurement rate of seven analyses per second is described for continuous CO<sub>2</sub> tension determination over the expiration time (or volume). The value recorded from the last portion of expired air is regarded as "the alveolar CO<sub>2</sub> tension" if a plateau has developed during expiration. Respiration in small animals is often so rapid (relative to the measurement rate) that forced prolongation of a single expiration is necessary in order to draw undiluted alveolar air into the analyzer. A simple method is described for accomplishing this by means of a valve whose closing is electronically initiated and timed.

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## 17112 P. Multiplication of the T3 Bacteriophage Against *E. coli*.

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Steps in the development of new bacteriophage particles from diseased bacteria are particularly well seen in electron micrographs of young and frequently transferred colon bacilli infected with the T2 strain of their bacteriophage.<sup>1</sup> Examination of such photographs has suggested that this bacteriophage is self-reproducing with a mode of multiplication that in some respects resembles that of coccoid bacteria. An unforeseen result of these observations has been the rapidity with which lysis, understood as rupture of the bacterial membrane, appears after infection with bacteriophage. It has usually been taken for granted that when a bacteriophage particle is adsorbed to a susceptible bacterium, it proliferates either at the surface or after penetrating the organism until, at the conclusion of the multi-

plication process, the cell bursts and frees the new particles that have been forming. Evidence from the electron micrographs demonstrates that the membranes of young colon bacilli in a growing culture rupture soon after mixture of the cells with enough T2 particles to disease them. On this basis the "burst interval" observed for these bacteriophages is determined by the dispersal of newly formed bacteriophage from the disappearing protoplasmic mass in which it has been developing.

Further light upon this and other aspects of the multiplication of bacteriophage has now been obtained by investigating the lysis of young *E. coli* by the two different bacteriophages, T1 and T3.

When a one-hour culture of the B strain of *E. coli* obtained by two or three transplants

<sup>1</sup> Wyckoff, Ralph, W. G., *Nature*, 1948, 162, 649.

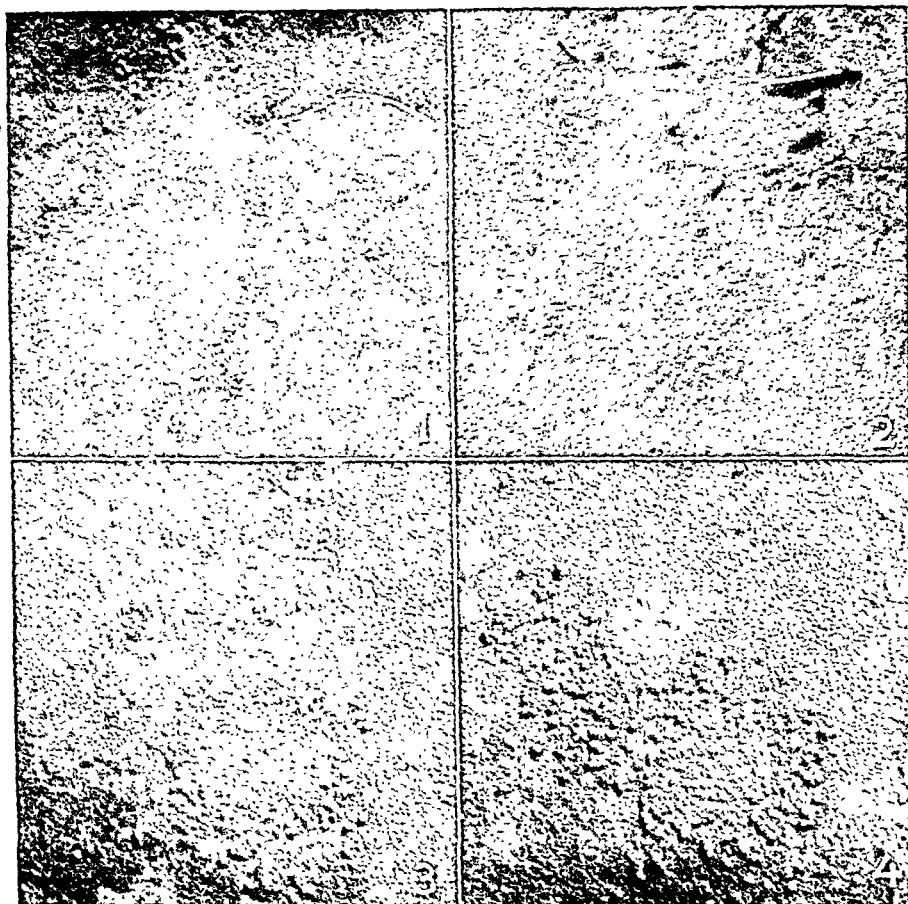


FIG. 1. A colon bacillus lysed through the action of T3 bacteriophage. From a culture after 5 minutes incubation. The cell membrane is at the right, the extruded granular protoplasmic contents at the left. Palladium shadowing on this and the following three preparations. Magnification, 16,667  $\times$ .

FIG. 2. Particles of T3 phage developing within the protoplasm of a lysed colon bacillus. The empty cell membrane is at the top of the picture. Magnification, 16,667  $\times$ .

FIG. 3. A somewhat more advanced stage in the development of a microcolony of T3 within protoplasm from a lysed bacterium. Magnification, 16,667  $\times$ .

FIG. 4. A still later stage of phage production in which most of the cellular protoplasm has been consumed and the new particles are beginning to disperse. Magnification, 16,667  $\times$ .

at hourly intervals has been mixed with enough T3 bacteriophage to infect at once all the bacteria present, electron micrographs have been obtained that agree with the earlier ones using T2 in showing prompt lysis; after 5 minutes incubation followed by immediate icing very few intact organisms have been found in centrifuged, formalinized and washed preparations. Such rupturing has not been observed in control preparations from which

the bacteriophage has been omitted. When T1 bacteriophage of the same titre has been substituted for T3 many, but far from all the young cells in a culture have been quickly lysed. As Fig. 1 indicates, the bacterial protoplasm liberated by these bacteriophages is the same mass of spherical macromolecular particles seen after lysis with T2.

Preparations made after incubating for intervals up to half an hour following infection

with T3 have shown various steps in the development of new bacteriophage particles in the protoplasm of infected and lysed cells. After 15 minutes incubation fields like those of Fig. 2, 3 and 4 are common. The lysed cell of Fig. 2 resembles that of Fig. 1 in showing its protoplasm extruded from the broken membrane. Much of this protoplasm has, however, already been converted into new particles of T3. The conversion is still more complete in Fig. 3 where the cluster of coccoid T3 apparently is developing from one or more central particles. This cluster recalls a bacterial microcolony resulting from several divisions of a single cell. A still later stage wherein the newly formed particles have commenced to disperse is shown in Fig. 4. After a few more minutes incubation the formation of bacteriophage has progressed so far at the expense of the lysed protoplasm that it is hard to find either the protoplasmic masses or the clumps of bacteriophage evident in these earlier photographs. Many electron micrographs like the foregoing have been obtained; they exhibit T3 particles in small groups and chains

that suggest they have arisen by the same kind of self-reproduction which is often seen in bacterial growths and which appears to underlie the multiplication of the T2 coli bacteriophage.

*Summary.* Cultures of young, rapidly growing susceptible colon bacilli obtained after hourly transplants to fresh broth appear quickly lysed by the addition of T3 bacteriophage. Electron micrographs show that the bacterial membranes rupture very soon after adsorption of bacteriophage and that colonies of new phage particles rapidly appear in the protoplasmic masses. This protoplasm from young cells is the same assemblage of spherical macromolecules seen after lysis with T2 phage. As was the case with this other phage the observed phenomena are those to be expected of particles behaving as if they were minute microorganisms multiplying by division at the expense of the bacterial protoplasm.

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### 17113. Effect of Chemical Irritation of the Peritoneum on Transmural Migration of Intestinal Organisms.

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For reasons already given elsewhere<sup>1</sup> it is believed that the peritonitis which develops during peritoneal irrigation for the treatment of acute renal failure is due to the transmural migration of bacteria from the intestine. While there is no evidence that this can occur in the absence of severe damage to the intestinal wall, it might be possible as a result of the combined effect of the uremic state and the irritating properties of the dialyzing fluid. In the absence of clinical evidence for or against such a hypothesis, animal experiments were performed to see if transmural migration of

intestinal bacteria can be induced (1) in normal animals subjected to chemical irritation of the peritoneum and (2) in normal and uremic animals exposed to the intraperitoneal injection of the fluids used for peritoneal irrigation in acute renal failure. The results of the first part of these studies are herewith reported.

*Method.* Healthy rabbits and dogs received sterile physiologic saline solution or the fluid used for peritoneal dialysis of uremic patients, in a volume sufficient to grossly distend the peritoneal cavity twice daily up to 2 weeks. Peritoneal fluid was aspirated as completely as possible just before each infusion. The as-

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<sup>1</sup> Frank, H., Seligman, A., Fine, J., *Annals Surg.*, 1948, 128, 3.

TABLE I.  
 Effect of Intraperitoneal Injection of Sterile Monolate Solution and of Sterile Gum Tragacanth Suspension in Rabbits and Dogs.

Day	Monolate.																				Gross pathology
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	
R <sub>1</sub> Injection (cc)	1	1	1	2	2		3	2	2	2	—	—	—	—	—	—	—	—	—	—	Fibrinous peritonitis.
R <sub>2</sub> Culture	—	—	—	—	+		+	+	+	+	+	+	—	—	—	—	—	—	—	—	
R <sub>2</sub> Injection (cc)	2	2	2	3	3	3	+	3	3	3						S					Fibrinopurulent peritonitis.
R <sub>3</sub> Culture	—	—	—	+	+		+	+	+	+	S										
R <sub>3</sub> Injection (cc)	1	2	2	2	2	2	+	2	2	2	3	3	3	3	3	3	3	3	3	3	Multiple adhesions. No active inflammation.
*R <sub>4</sub> Culture	—	—	—	+	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+	
*R <sub>5</sub> Injection (cc)	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	" " " " " "
*R <sub>5</sub> Culture	—	—	—	—	—		4	4	4	4	4	4	4	4	4	4	4	4	4	4	
*R <sub>6</sub> Injection (cc)	2	3	3	3	3	3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	" " " " " "
*R <sub>6</sub> Culture	—	—	—	+	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+	
*R <sub>7</sub> Injection (cc)	2	3	3	3	3	3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	" " " " " "
*R <sub>7</sub> Culture	—	—	—	+	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+	
D <sub>1</sub> Injection (cc)	4	5	5	5	5	5	8	8	—	5	5	5	5	5	5	—	—	—	—	—	Fibrinopurulent peritonitis.
D <sub>2</sub> Culture	—	—	—	—	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
D <sub>2</sub> Injection (cc)	5	5	5	5	5	5	5	—	—	—	—	—	—	—	—	—	—	—	—	—	" " " " " "
D <sub>3</sub> Culture	—	—	—	+	+		+	+	S												
D <sub>3</sub> Injection (cc)	5	5	5	5	5	5	5	5	—	—	—	—	—	—	—	—	—	—	—	—	" " " " " "
D <sub>3</sub> Culture	—	—	—	+	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+	
R <sub>1</sub> Injection (cc)	10	10	—	—	—				+	+	+	+	+	+	+	+	+	+	+	+	Fibrinopurulent hemorrhagic peritonitis.
R <sub>2</sub> Culture	—	—	—	—	—				+	+	+	+	+	+	+	+	+	+	+	+	
R <sub>2</sub> Injection (cc)	10	—	+	+	+	+	S														Subacute peritonitis.
R <sub>3</sub> Culture	—	—	—	+	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+	
R <sub>3</sub> Injection (cc)	10	—	—	—	—				+	+	+	+	+	+	+	+	+	+	+	+	Fibrinopurulent peritonitis.
R <sub>3</sub> Culture	—	—	—	+	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+	
D <sub>1</sub> Injection (cc)	40	40	40	40	40	40	40	40	—	—	—	—	—	—	—	—	—	—	—	—	Fibrinous peritonitis.
D <sub>2</sub> Culture	—	—	—	—	—				—	—	—	—	—	—	—	—	—	—	—	—	
D <sub>2</sub> Injection (cc)	40	40	40	40	40	40	40	40	—	—	—	—	—	—	—	—	—	—	—	—	" " " " " "
D <sub>3</sub> Culture	—	—	—	+	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+	
D <sub>3</sub> Injection (cc)	40	40	40	40	40	40	40	40	—	—	—	—	—	—	—	—	—	—	—	—	" " " " " "
D <sub>4</sub> Culture	—	—	—	+	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+	
D <sub>4</sub> Injection (cc)	40	40	40	40	40	40	40	40	—	—	—	—	—	—	—	—	—	—	—	—	

The volume of solution injected each day is indicated by the number. Blank spaces prior to S indicate that no injection or culture made on that day.  
 R = Rabbit. D = Dog. — = Sterile culture. + = Presence of *E. coli* in peritoneal fluid. S = Sacrifice of animal.  
 \* R<sub>1</sub>—Cultures positive > 6 days, followed by 8 negative cultures and sacrifice of rabbit on 36th day. R<sub>5</sub>, R<sub>6</sub>, R<sub>7</sub>—Cultures negative > 9 days and sacrifice of rabbits on 32nd day.

pirated fluid was always found devoid of bacteria by aerobic and anaerobic culture. A moderate leukocytic response was regularly present after the first few infusions. Post-mortem examination of some of the animals after 2 weeks showed no grossly recognizable changes in the peritoneum.

A severe inflammation of the peritoneum was then produced in a second group of animals by the intraperitoneal injection of sterile 5% monolate (monoethanolamine oleate), which is strongly irritating to endothelial cells. Since a single dose of 4 cc of monolate killed rabbits within a few hours, a graded dose of from 1-4 cc was injected daily into each of a series of rabbits. Each of a series of dogs received 5 cc of monolate daily.

Another series of rabbits and dogs received a daily dose of 5 cc and 40 cc of sterile 2½% gum tragacanth suspension<sup>2,3</sup> respectively, for a number of days. Samples of peritoneal fluid were taken daily, with sterile precautions, from all animals. The animals were sacrificed on the day following the last injection or at some later date. When the tap was dry, 5 or 10 cc of sterile saline were injected and, after a few minutes, a portion of this fluid was withdrawn. All specimens were smeared and cultured immediately in tryptone broth, on agar and on endoplates. The same small loop was used for all inoculations, so that positive plates would permit a roughly quantitative measure of the degree of contamination. Broth and plates were observed for as long as 10 days.

Contamination of peritoneal fluid from the irritant itself or from the skin at the site of puncture was avoided by sterile technic and by the finding of no growth in cultures made from the irritant and from the skin. Daily blood cultures were also done on several animals throughout the experiment to exclude bacteremia as a source of peritoneal contamination. Identification of bacteria was made by standard bacteriologic technics.

*Results Table I.* Cultures of all animals injected with monolate or gum tragacanth if

<sup>2</sup> Steinberg, B., Goldbatt, H., *Int. Med.*, 1927, 30, 449.

<sup>3</sup> Steinberg, B., *Am. J. Clin. Path.*, 1936, 6, 253.

positive contained *Escherichia coli* and never any other organism. The number of colonies on direct plates was always less than 50 colonies per plate. *E. coli* was found regularly in the peritoneum of all rabbits and dogs beginning on the 4th to 9th day of injection. Once the microorganism appeared, it continued to be present until the injections were stopped. In four rabbits the culture remained positive for from 3 to 7 days after the last injection, and sterile for from 9 to 14 days thereafter. These rabbits showed extensive intraperitoneal adhesions without active inflammation, while those sacrificed shortly after the last injection showed diffuse fibrinopurulent and adhesive peritonitis. That the fibrinopurulent peritonitis was produced by the monolate rather than by *E. coli*, is indicated by finding in several rabbits and dogs sacrificed after only 2 or 3 days of monolate injections that cultures taken each day and at postmortem were sterile despite the presence of a marked fibrinopurulent peritonitis.

*Gum-tragacanth Experiment (Table I):* Three rabbits injected intraperitoneally with one dose of gum-tragacanth showed a fibrinopurulent peritonitis within 24 hours and *E. coli* was recovered at the same time. In dogs, however, the results were somewhat different. Four dogs, injected once, revealed sterile peritoneal cultures each day up to the time when they were killed on the 2nd, 4th, 5th, and 7th days respectively. At autopsy they all showed mild degrees of fibrinopurulent or fibrinous peritonitis. The results were the same in 2 more dogs which received daily doses of tragacanth for 4 days and for 7 days respectively. In another which received a daily dose of 40 cc of tragacanth for 6 days, *E. coli* first appeared on the 4th day and continued to be present in the peritoneal exudate for 4 more days. At postmortem he showed severe fibrinopurulent peritonitis. In still another dog similarly treated for 4 days *E. coli* was first found on the 5th day and continued to be present for 4 more days, after which sterile cultures were obtained during 5 subsequent days. Autopsy revealed a resolving fibrinous peritonitis. That the positive cultures were in no instance due to bacteremia, contamination from the skin at the

site of injection or of the irritant itself was shown by uniformly negative cultures from these sources.

*Discussion.* In spite of the large number of investigations which have been made on the permeability of the intestinal wall for bacteria, conclusive evidence of transmural migration of intestinal bacteria has not been produced. A normal intact serosa is generally regarded as impermeable, but this may not be the case where there is inflammation of the peritoneum due to a foreign body or a chemical irritant. For example, the presence of bile or a sponge in the peritoneal cavity may result in peritonitis or abscess formation, with organisms of intestinal origin present in the absence of a visible break in the continuity of the intestinal serosa. However, convincing proof that in such instances no break in continuity, however small, was responsible is difficult to produce.

Experimental data are contradictory. When a foreign substance, such as melted agar or sodium nucleinate<sup>4</sup> or broth<sup>5</sup> is placed in a serous cavity, intravenously injected cocci are found occasionally in the cavity containing the foreign substance but not in the other cavities. Thus an irritant in the peritoneal cavity may attract and retain intravenously injected cocci, but such organisms obviously must have entered the cavity via the circulation. Peritonitis following the feeding of virulent cocci usually does not occur.<sup>6-12</sup> In several instances where this did happen transmural migration was not proved, because bacteria may have entered the circulation via the inflamed intestinal wall.<sup>13-17</sup>

The recovery of the organism responsible for primary peritonitis from the intestinal wall<sup>18,19,16</sup> or in the stool<sup>20,21</sup> does not establish the case for transmural migration, because all of the involved areas may have been invaded from the blood stream. The evidence that the intestinal serosa when intact can be traversed by bacteria has therefore been equivocal.

In our experiments the sterility of the injected substance and of the skin, ascertained by daily cultures, exclude an external source for the bacteria found in the peritoneal cavity of our animals. Invasion via the blood stream is unlikely. To exclude this remote possibility daily blood cultures from two rabbits and one dog were taken during the entire time of the experiment and yielded no growth. In view of these observations the regular finding of a typical intestinal microorganism in pure culture in the peritoneum inflamed by chemical irritation is strongly in favor of its origin from the gut by transmural migration, in spite of the absence of any signs at postmortem examination of mucosal inflammation. Why only *E. coli* and none of the other intestinal bacteria was recovered is difficult to say. Pure *E. coli* peritonitis is rare.<sup>22,23,13</sup> The fact that *E. coli* was the usual contaminant or infecting organisms in patients subjec-

<sup>13</sup> Lenander, K., Nyström, G., *Z. f. Klin. Med.*, 1906, **63**, 263.

<sup>14</sup> Geirssvald, S., quoted by Lenander, K. G., Nyström, G.<sup>13</sup>

<sup>15</sup> Felsen, J., Osofsky, A. G., Pharyngogenic Hematogenous Streptococci Peritonitis, *Arch. Surg.*, 1935, **31**, 437.

<sup>16</sup> Weil, S. A., Saphir O., *Am. J. Dis. Child.*, 1932, **43**, 611.

<sup>17</sup> Montgomery, A. H., *Surg. Gyn. Obst.*, 1925, **21**, 798.

<sup>18</sup> Escherich, T., *Jahrb. f. Kinderheilkunde*, 1889, **40**, 167.

<sup>19</sup> Flexner, S., *The Johns Hopkins Hospital Bull.*, 1895, **6**, 64.

<sup>20</sup> Obadalek, W., *Deutsche Z. f. Chir.*, 1929, **220**, 307.

<sup>21</sup> Obadalek, W., *Deutsche Z. f. Chir.*, 1931, **233**, 587.

<sup>22</sup> Welsh, quoted by Flexner.<sup>19</sup>

<sup>23</sup> Fishbein, M., *Am. J. Med. Sciences*, 1912, **144**, 502.

<sup>4</sup> Pawlowski, P., *Zentralblatt f. Chir.*, 1887, No. 48; *Virchows Arch.*, vol. 117, 1889, quoted by Künzel, H., *Münchn. Med. Woch.*, 1904, **51**, 1920-1921.

<sup>5</sup> Peiser, A., *Beitr. z. Klin. Chir.*, 1907, **55**, 484.

<sup>6</sup> Austerlitz, F., Landsteiner K., *Sitzungsberichte der kaiserlichen Akademie der Wissenschaften in Wien*, 1889.

<sup>7</sup> Markus, H., *Z. f. Heilkunde*, 1899, **29**, 427.

<sup>8</sup> Neisser, M., *Z. f. Hyg.*, 1906, **54**, 363.

<sup>9</sup> Opitz, E., *Z. f. Hyg.*, 1904, **48**, 67.

<sup>10</sup> Selter, H., *Z. f. Hyg.*, 1896, **22**, 12.

<sup>11</sup> Bail, M., *Arch. f. Klin. Chir.*, 1900, **62**, 369.

<sup>12</sup> Hornemann, E., *Z. f. Hyg.*, 1911, **69**, 39.



ted to peritoneal irrigation for uremia suggests that the mechanism of invasion in these patients was similar to that in the foregoing experiments.

**Summary.** Chemical peritonitis induced by repeated injections of monolate or gum-tragacanth in rabbits and dogs results in the appearance of *E. coli* in pure culture, in small to moderate numbers, in the inflamed peritoneum. Once this contamination is established,

it continues for some time after the injections are discontinued. Eventually all cultures are sterile.

By exclusion of all other possible sources of contamination of the inflamed peritoneum, it appears that the *E. coli* found in the peritoneal exudate is derived from the intestinal lumen by transmural migration.

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### 17114. Transmural Migration of Intestinal Bacteria During Peritoneal Irrigation in Uremic Dogs.\*

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Peritonitis due to *Escherichia coli* in pure culture occurs readily during peritoneal irrigation for the treatment of acute renal failure.<sup>1</sup> Clinical data suggested that the peritonitis was due to invasion of the peritoneum by the transmural migration of *E. coli* from the intestinal lumen.<sup>1</sup>

In a previous experimental study<sup>2</sup> it was demonstrated that transmural migration of *E. coli* can be induced by the intraperitoneal injection of strong irritants, but that bacterial peritonitis does not ensue. It was then considered desirable to determine whether the irrigating fluid used in treating acute renal failure can also provoke a bacterial invasion of the peritoneal cavity from the gut. The data to follow demonstrate that bacterial contamination is produced following intraperitoneal administration of this irrigating fluid in uremic

dogs but not in normal dogs and that prophylactic oral antibiotic therapy is effective in preventing it.

**Method.** Normal dogs, and dogs made uremic by removal of both kidneys 24-72 hours previously, were treated as follows: Fifteen hundred to 3000 cc of sterile irrigating fluid‡ was injected into the peritoneal cavity by needle puncture under rigid sterile precautions. The fluid, as it escaped from the needle, just before and just after the puncture, was inoculated into dextrose broth. At the same time, swabs of the site of puncture of the skin, which had been prepared by shav-

‡ The 2 solutions used for infusion were those which had been used in patients for peritoneal irrigation.<sup>1</sup> Each was used in about half of the experiments. As there was no difference in their effect, the results are not considered separately.

\* The work described in this paper was done under a contract, recommended by the Committee on Medical Research of the Office of Naval Research of the United States Navy and Harvard University.

† With the technical assistance of Sunya Gordon and Dorothy Kaufman.

1 Frank, H. A., Seligman, A. M., and Fine, J., *Ann. Surg.*, 1948, **128**, 561.

2 Schweinburg, F. B., and Heimberg, F., *Proc. Soc. Exp. Biol. and Med.*, 1949, **71**, 146.

	Solution A (g/l)	Solution B (g/l)
NaCl	7.25	7.4
KCl	0.19	0.05
CaCl <sub>2</sub>	0.095	0.05
MgCl <sub>2</sub> · 6H <sub>2</sub> O	0.2025	0.22
NaHCO <sub>3</sub>	1.5	0.77
Dextrose	9.5	5.0
Gelatin§	4.75	16.0
pH after sterilization	7.9	7.2-7.6

§ We are indebted to the Atlantic Gelatin Company for the gelatin used in these experiments.

TABLE I. Summary of Experiments.

Dog No.	Preliminary treatment	Infusion, days	Total vol. infused (cc) $\times 1000$	Organisms in peritoneal fluid*			Cause of death	Gross peritonitis
				<i>E. coli</i> , day	Others			
1	None	11	10.5	0	0		Distemper	No
2	"	13	16.9	0	0		Sacrificed	"
3	"	16½	40	0	0		"	"
4	Bilateral nephrectomy	9	10	6‡	0		Uremia	"
5	"	4	9.4	0	0		Pulmonary edema	"
6	"	6	11.4	5	<i>A. aerogenes</i>		Uremia	"
7	"	9	19.2	7	"		"	"
8	"	11	30.1	9	"		"	"
9	"	14	44.9	7	"		"	"
10	"	3	7.2	0	<i>Paracoli</i>		"	"
11	"	5½	10.5	6	<i>A. aerogenes</i>		"	Fibrinopurulent peritonitis
12	"	9	37.8	12	<i>B. pyocyaneus</i>		"	No
13	"	4	11.8	4	<i>A. aerogenes</i>	enteritis	"	Diffuse hemorrhagic peritonitis
14	"	13	39.5	7	<i>B. megatherium</i>	"	"	No
15	"	8½	39.2	8	0	"	"	No; no foreign body reaction
16	"	2	5.5	0	0	Pneumonia	"	Fibrinous adhesions about foreign bodies
17	"	4½	14.5	3	<i>Strep. fecalis</i>	Uremia	"	No; no foreign body reaction
18	Sulfathalidine and streptomycin orally 5 days, then bilateral nephrectomy	6	18.5	0	0	"	"	No
19	Sulfathalidine and streptomycin orally 4 days, then bilateral nephrectomy	5	17.8	0	0	"	"	"
20	Same	12	44.7	0	0	Sacrificed	"	"
21	Same	9	38.6	0	0	Uremia	"	"

\* Organisms, when present, persisted from the day first found until death, with the exception of inconstant growth of *A. aerogenes* in Dog 9.

† Days before start of infusions.

‡ Days after start of infusions.

ing, alcohol and zephiran, were also inoculated into dextrose broth. To establish the initial sterility of the peritoneal cavity, fluid aspirated from the peritoneal cavity shortly after the first infusion was inoculated into dextrose broth, on agar and endo plates and into anaerobic media. Infusions were given twice daily and the foregoing procedures were repeated on each occasion, except that just preceding each subsequent infusion all the fluid which could be aspirated was removed and a sample inoculated into the various media. Cultures were observed for 10 days unless they became positive earlier.

Three normal dogs (No. 1-3) received a total of 10-40 liters in a period of 11-16 days. In 2, very little of the infused fluid was recovered by aspiration, but in the third, 30 of the 40 liters given were recovered.

Eighteen dogs were made uremic by removal of both kidneys. They are divided into 3 groups. The first group of 11 (No. 4-14) was treated in the same way as the normal dogs, but the food and fluid offered postoperatively was not consumed. In the second group of 3 (No. 15-17) a sterile foreign body (a rubber or stainless steel tube or both) was placed in the peritoneal cavity at the time of nephrectomy. In the third group of 4 (No. 18-21) 0.10 g of streptomycin and 1.0 g of sulfathalidine were given orally for 4-5 days prior to nephrectomy. Daily aerobic and anaerobic cultures of the stool were prepared and observed for several days preceding nephrectomy.

Blood was taken for culture from all dogs every second day until death.

**Results (Table I) Normal Dogs.** In all 3 normal dogs a few leucocytes per high power field appeared in the peritoneal fluid for the first time on the third day of the infusions. They increased only slightly in number thereafter. The peritoneum at the end of the experimental period was smooth, glossy, and entirely free of any reaction. No growth was obtained in any of the peritoneal fluid cultures. It is, therefore, evident that exposure of the peritoneal cavity of a normal dog to these irrigating fluids for much of each day

for a period of some 2 weeks does not produce detectable bacterial contamination.

In 10 rabbits treated and observed in the same way for 14-16 days, the results were precisely the same.

**Uremic Dogs.** Untreated dogs survive not more than 3-4 days after bilateral nephrectomy. Two of the 11 dogs in Group I (No. 4-14) died on the 3rd and 4th day of infusion respectively. The aspirated fluid remained sterile. In the remaining 9, the aspirated fluid was sterile for the first 3 days of infusion. Leucocytes in the aspirated fluid appeared for the first time on the 2nd or 3rd day, and increased only slightly in number thereafter. Positive cultures were obtained for the first time from the fourth to the ninth day of infusion, and remained positive in all 9 dogs until death, which occurred 5-16 days after the infusions were started. *E. coli* was the contaminating organism in all instances; in pure culture in 2, together with *Aerobacter aerogenes* in 7. The number of colonies was always small, with *E. coli* predominating, except in Dog. 11. No clostridia were found in any of the anaerobic cultures. Only 2 dogs (No. 11 and No. 13) developed purulent peritonitis. In Dog 11, *E. coli*, *A. aerogenes* and *Pseudomonas aeruginosa* (*B. pyocyaneus*) were isolated. In Dog 13, *E. coli* and *Bacillus megatherium* were isolated.

In Group II (Dogs 15-17), contamination appeared for the first time on the 8th day of infusion in Dog 15, and on the 3rd day in Dog 17. Peritonitis did not develop in these dogs. Dog 16, which died of pneumonia 72 hours after nephrectomy, showed a sterile peritoneal inflammation after 48 hours of infusion. It appears that the sterile foreign body does not influence the time or degree of invasion of the peritoneal cavity by intestinal organisms.

In Group III (Dogs 18-21), the standard stool cultures remained sterile. A fecal suspension three times as dense as the standard yielded scanty growth of *Streptococcus fecalis* and diphtheroids, but no *E. coli* or clostridia. None of the four dogs in this group showed bacterial contamination of the aspirated fluid at any time, although the leucocyte response was the same as in the other

|| Anaerobic cultures were done in 6 of the uremic dogs.

nephrectomized dogs. Their survival times were 5, 6, 9 and 12 days after the start of the infusions.

Death was due to uremia in all dogs in all 3 groups (except Dog 16) with purulent peritonitis contributing in Dogs 11 & 13.

The blood cultures were uniformly sterile. Cultures from the skin of the abdomen and of the irrigating fluid were always sterile before the infusion. Exceptionally a scanty growth was obtained from the skin after the infusion was given. The organisms recovered were the usual skin inhabitants, *i.e.* *Staphylococcus albus*, *Bacillus subtilis*, diphtheroids or sarcinae, and not the intestinal flora recovered from the aspirated fluid (Table I).

**Discussion.** The bacteria recovered from the aspirated fluid are clearly of intestinal origin. 1, because of the species found and 2, because their elimination from the intestine by oral chemotherapy prevents peritoneal contamination. The route of invasion is transmural, since the blood cultures were negative. The number of bacteria recovered in cultures was always small. Presumably this number is a measure of the balance between the number invading and the number leaving or being destroyed. It has been observed<sup>3</sup> that the peritoneum of dogs can destroy or eliminate a large number of bacteria without reacting with severe inflammation. Since the peritoneal cultures were uniformly negative in normal irrigated dogs, it appears that the uremic state makes the intestinal wall more permeable to bacteria or reduces the ability of the peritoneum to destroy them. The fact that only two of the fourteen uremic dogs which showed invasion developed purulent peritonitis may be due to the small number of invading bacteria or to the considerable natural resistance of the dog to these organisms.

It is noteworthy that the organisms recovered from the uremic dogs were the same as those from uremic patients treated with the same irrigating fluid.<sup>1</sup> Since the leucocyte response to the fluid in the normal animal is very slight, the fluid is at most a very mild stimulus to inflammation. Since neither the irrigating

fluid alone nor uremia alone<sup>1</sup> is capable of producing peritoneal contamination, it appears that both factors acting together are responsible for the bacterial contamination. The precise role played by the uremic state is not clear. The fact that many of the uremic dogs showed an edematous intestinal wall at autopsy suggests that transmural migration may be due to the edema resulting from the absence of renal excretion, and that the uremia itself, so far as this phenomenon is concerned, is merely coincidental. However, in our clinical experience, the incidence of peritoneal contamination in irrigated patients was no less in those in whom overhydration was avoided. The ready development of *E. coli* peritonitis in man following contamination, as compared to animals, may be due to a lower natural resistance to *E. coli*, which the uremic state may further reduce.

This experimental study suggests that the treatment of acute renal failure in man by peritoneal irrigation may prove less hazardous, if a sufficient reduction of intestinal bacteria can be accomplished before treatment and maintained during treatment.

**Summary and conclusions.** Repeated intraperitoneal infusion of the fluid used for peritoneal irrigation in uremic patients, administered aseptically to healthy rabbits and dogs for 11-16 days, produced a moderate leukocytic response, but the peritoneum remained free of bacteria or visible inflammatory reaction. In uremic dogs, however, bacterial contamination of the peritoneal fluid developed after the 4th day of irrigation. The micro-organisms found were *E. coli* alone or with *A. aerogenes*. Occasionally, several other species, normally found in the intestine, appeared: but clostridia were never found. Preliminary oral administration of streptomycin and sulfathalidine prevented contamination of the peritoneal fluid. Blood cultures were uniformly sterile throughout the period of observation. It is concluded that irrigation of the peritoneal cavity of uremic dogs causes transmural migration of intestinal bacteria and that oral chemotherapy can prevent such contamination.

<sup>3</sup> Steinberg, B., Goldblatt, H., *Arch. Int. Med.*, 1927, 39, 446; 1927, 39, 449.

# 17115. Experimental Studies on Nutrition in Tuberculosis. The Role of Protein in Resistance to Tuberculosis.\*†

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The view is widely prevalent, and well supported by demographic evidence, that a high level of dietary protein is protective against invasion by tuberculosis.<sup>1-3</sup> Recent experimental and other evidence has shown that protein metabolism is of basic importance in natural and acquired resistance to infection in general,<sup>4</sup> and shown that it may be of importance in tuberculosis.<sup>5</sup> The investigation here recorded was carried out to determine if a difference in susceptibility to tuberculosis can be detected in experimental animals nourished on diets of variable protein content.

In the stock of albino rats at the Wistar Institute of Anatomy and Biology, at the time these experiments were initiated, there were 3 colonies of animals which had lived for a number of generations on diets containing respectively 15, 25 and 40% of protein. Metabolic and growth studies on these animals have been reported by McCoy,<sup>6</sup> who found that rats raised on a diet containing 40% protein grew more rapidly and were more prolific than rats on diets with smaller protein content. Appearance and behavior differences were striking. The animals on a diet of 15% protein were sluggish and sleepy,

while those on the 40% diet were active and playful. The former had ruffled and the latter sleek and shining hair. The 25% animals were between the two extremes.

The base diet furnished these animals was composed as follows:

	g
Whole wheat flour	3675
Skimmed milk powder	800
Whole milk powder	1200
Soy bean meal	400
Alfalfa leaf meal	300
Irradiated yeast	300
Dried liver	200
Calcium carbonate	25
Bone ash	80
Sodium chloride, iodized	20
	<hr/> 7000

From this base, 15, 25, and 40% diets were prepared as follows:

Composition of Diet, and Protein Level in Per Cent.

	15	25	40
Base	4200 g	4200 g	4200 g
Cod liver oil	240	240	240
Dextrin	1560	960	—
Casein	—	600	1560
	<hr/> 6000	<hr/> 6000	<hr/> 6000

Animals from the 3 groups were used for the experiments here recorded. Approximate equalization was effected within each protein group of experimental animals as respects age, weight and sex. All animals were injected intravenously with virulent tubercle bacilli of bovine type. Two separate experiments were carried out. The dosage was more accurately calibrated in the second than the first. In the first, animals of approximately equal weight were selected at the start and each received 0.1 mg tubercle bacilli in the internal jugular vein. In the second experiment, the injection carried out by the same route was so regulated that each animal received approximately the same dosage per unit lung weight. Lung weights were esti-

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† A preliminary report was presented at the IV International Congress for Microbiology, Copenhagen, Denmark, July 20-26, 1947.

<sup>1</sup> Faber, K., Tuberculosis and Nutrition, *Acta Tuberc. Scandinarv.*, 1938, **12**, 287.

<sup>2</sup> Kirschner, M., *Z. f. Tuberk.*, 1921, **34**, 228.

<sup>3</sup> Schröder, G., *Beitr. z. Klin. d. Tuberk.*, 1930, **75**, 61.

<sup>4</sup> Cannon, P. R., *J. Am. Med. Assn.*, 1945, **128**, 360; *Some Pathologic Consequences of Protein and Amino Acid Deficiencies*, Charles C. Thomas, Springfield, Ill., 1948.

<sup>5</sup> Ratcliffe, H. L., *Am. Rev. Tuberc.*, 1946, **54**, 389.

<sup>6</sup> McCoy, R. H., *J. Biol. Chem.*, 1940, **133**, lxiv.

TABLE I.  
Tuberculosis in Rats on Diets with Varying Percentage of Protein (Exp. 1).

% protein	Days of life	No. animals	Extent tuber.	Predom. type cellular react.	Avg degree localization	Avg No. bacilli	% with pneumonia
15	78-150	10	2.4	Epithelioid	Poor	Large	30
	167	2	3.0	"	"	"	100
25	47-150	9	2.7	"	"	"	44
40	78-150	5	2.4	"	"	Moderate	40
	196-293	7	2.4	Regr. mono.	"	"	100

TABLE II.  
Tuberculosis in Rats on Diets with Varying Percentage of Protein (Exp. 2).

% protein	Days of life	No. animals	Extent tuber.	Predom. type cellular react.	Avg degree localization	Avg No. bacilli	% with pneumonia
15	14-150	10	2.1	Prog. mono.	Poor	Small	60
	180-222	4	1.8	Epithelioid	Moderate	"	25
	253-315	4	2.5	"	Poor	Moderate	50
25	14-150	4	1.5	"	"	"	50
	180-222	1	2.0	Regr. mono.	"	"	0
40	14-150	8	1.4	Prog. mono.	Good	Small	25
	180-222	6	1.5	Epithelioid	"	"	50
	253-315	10	1.3	Regr. mono.	Fair	"	20

mated from a formula worked out at the Wistar Institute.<sup>7</sup>

Animals were autopsied as they died or were killed at selected intervals. Sections were stained by hematoxylin and eosin, and also with carbol-fuchsin, by the technic for acid-fastness, in order to show the number of tubercle bacilli in tuberculous lesions. The most significant facts learned were with reference to pulmonary lesions, and the following analysis is based on the development of tuberculosis in the lungs.

A simple scoring system was adopted, which was based on (a) the extent of the lesion, (b) the type of cellular reaction, (c) the degree of localization of the tuberculous process and (d) the average number of tubercle bacilli present in lesions. In addition, because of what proved a significant development in the experiment, notation was made as to the presence or absence of extensive pneumonic lesions at the time of death. The extent of tuberculosis was estimated on a simple basis as follows: 1 = slight; 2 = moderate; and 3 = extensive.

The results of the 2 experiments are indicated in Tables I and II.

Table I illustrates the results of the first experiment, in which 33 rats, matched according to age and litter, were used. This group of animals developed pneumonia in a high percentage of cases. All of the animals on 15 and 25% protein, including those sacrificed, died within less than 168 days. Animals were sacrificed for comparable observation on the 78th and 150th day of the experiment. Eight rats on the diet containing 40% protein outlived all of the other animals, although all but one of these died from natural causes ultimately. The last animal of the group was killed on the 293rd day.

Tuberculosis developed rapidly in all animals and the extent exceeded "moderate" in all within the first 150 days. In the animals on the 15% protein diet very extensive tuberculosis occurred after the 150th day. After the 150th day in the animals on 40% protein little or no progression occurred, and in most animals a certain amount of regression developed, evident both by decrease in the size and extent of the lesion and by a tendency to regression in the type of reaction.

In general in the earliest days of infection

<sup>7</sup> Donaldson, H. H., The Rat, Memoirs of the Wistar Institute of Anatomy and Biology, No. 6, 1924, p. 212.

a type of cellular reaction was evident which is here designated as a progressive mononuclear response. It was characterized by the early appearance of large mononuclear cells of the macrophage series with large nuclei and a small amount of cytoplasm. With the passage of time these mononuclear cells phagocytized tubercle bacilli and developed a large amount of cytoplasm, taking on the appearance of large epithelioid cells (epithelioid stage). The average type of reaction in all the animals up to the 150th day of the experiment was epithelioid. The 15 and 25% animals did not live long enough to develop the characteristic regressive type of mononuclear reaction. This was conspicuous, however, in the 40% animals, which lived much longer. This type of reaction was seen in most animals living longer than 180 days, and was particularly frequent in the animals receiving the highest concentration of protein. In this stage large mononuclear cells with a small amount of cytoplasm again became predominant and were present in far larger numbers than the typical epithelioid cells. Two explanations are possible for this phenomenon, (1) that the epithelioid cells gradually destroyed tubercle bacilli, after which the cytoplasm shrank, and (2) that epithelioid cells containing large numbers of tubercle bacilli were themselves destroyed and autolyzed, and replaced by new infiltrating large mononuclear cells, which found relatively few bacilli to phagocytize, and therefore did not develop the large amount of cytoplasm characteristic of epithelioid cells.

In the animals on diets containing 15 and 25% protein tubercle bacilli were present in enormous numbers. In the 40% animals the number of bacilli present was definitely smaller.

In the 3 groups of animals in Experiment I, nourished on varying amounts of protein, significant differences were not evident in the incidence of pneumonia. In the first 150 days from 30 to 40% of all animals developed this disease and died. The few remaining 15% animals died of pneumonia at 167 days. The 40% animals lived many months longer, but all but the one finally killed ultimately suc-

cumbed to pneumonia.

Characteristic differences occurred in the weight curves of the 3 groups of animals. These curves are not plotted in this paper, because of the complication introduced by the fact that certain animals were killed, and the fact that the weights of others were irregularly influenced by the development of pneumonia. The general characteristics of the curves, however, were as follows: Animals on the 15% protein diet increased in weight from an average of approximately 190 g at the beginning of the experiment to approximately 280 g at about the 90th day, and decreased to approximately 220 g at the 150th day. Animals of the 25% series increased from an original average weight of approximately 200 g to an average of about 300 g on the 90th day and dropped to an average of 255 g on the 133rd day. Two surviving animals weighed approximately 310 g when killed at 150 days. Animals of the 40% series increased from an average of approximately 210 g at the start to about 300 g at the 84th day, and held this until the end of the experiment.

In summary it may be said of the animals of Experiment I that the extent of tuberculosis did not vary greatly in the 3 groups. However, the animals on the 40% protein diet outlived those on diets containing lower percentages of protein, by several months, and the number of tubercle bacilli visible in the lesions was much smaller than in the animals on 15 and 25% protein diets. At times in the 15% animals the number of bacilli was enormous, and wide dissemination of bacilli was evident in some of the animals dying of pneumonia. In a number of the animals with pneumonia ulceration of bronchi by the pneumonic process and wide scattering of exudate appeared to have a definite effect in spreading tubercle bacilli.

In Table II the results of the second experiment are recorded. Forty-seven rats were used. As noted above, the dosage was accurately measured on the basis of lung weight. The extent of tuberculosis in all groups was less than that in the preceding experiment, and differences in degree of localization were more clearly evident.

In the tabulation of Experiment II the animals are divided into 3 groups, *viz.*, those dying between 14 and 150 days, those dying between 180 and 222 days, and those dying between 250 and 315 days.

The extent of tuberculosis was measured on the same scale as in the case of the animals recorded in Table I. The extent of tuberculosis was somewhat greater in the animals receiving 15 and 25% protein diets than in those on the 40% diet. Also, as in Experiment I, after the first 150 days significant increases in extent of tuberculosis did not develop in the 40% animals.

In general the animals of this experiment showed the characteristic change in cellular reaction noted in Experiment I. This was most typical in the 40% animals, which clearly exhibited a progressive mononuclear reaction up to 150 days as the predominant type of reaction, followed by a long period during which the overwhelming majority of cells present in the exudate were of the epithelioid type, with a final period, after 250 days, when the epithelioid cells diminished in number, and were replaced by large mononuclear cells with relatively little cytoplasm. The latter stage, as noted above, is designated in this paper as regressive mononuclear stage.

In this experiment, in contrast to the first, distinct variations in the degree of localization of the tuberculous lesions were noted. In general the disease was widely disseminated in the 15 and 25% protein animals, but a "fair" to "good" degree of localization was evident in animals on the 40% protein diet. The number of bacilli visible in the tuberculous lesions was smaller in this experiment than in the previous one, but, as in that experiment, the number seen in tuberculous lesions was definitely smaller in the 40% animals than in those receiving smaller amounts of protein. Highly suggestive differences in the extent of pneumonia in the animals of the three groups were observed. The incidence of pneumonia was very high in the 15% group, and, while still high in the 40% group, it was much below that found in the 15% group. The difference in frequency and extent of pneumonia was more

closely correlated with the amount of protein in the diet than was the extent of tuberculosis. The impression was created that, if the diet was responsible for variation in tuberculosis, the influence was indirect, through an effect upon resistance to the common types of pneumonia endemic in rat colonies. Bacteriological studies of the pneumonic lesions were not made. Previous studies on rats of this colony showed pneumonia is frequent and the etiology variable.<sup>8</sup>

As in the preceding experiment, in general the 40% animals far outlived animals of the other groups. At 315 days 5 of the 40% animals which had survived, were killed to conclude the experiment. These were large animals, free from pneumonia, with one exception, with excellent coats of hair, and every appearance of good health. As noted, all of them had moderately extensive tuberculosis, but this appeared to be in the regressive stage, as indicated by the type of cellular reaction.

Weight curves were comparable to those of the preceding experiment. In Experiment II, young and old animals were used, evenly distributed within the 3 groups. Young litters ranged from 61 to 76 days of age at the time of injection, and old litters from 736 to 931 days. All of the old animals died within the first 146 days. The animals which survived 315 days, all of them within the 40% group, were from 61 to 76 day litters. Differences in the type of cellular reaction were not observed between young and old animals.

*Summary.* The results of Experiment II were like those of Experiment I, the chief difference being that the infection was better regulated, and variations within the 3 protein groups were more characteristic. Tuberculosis was less extensive and life was much longer in the rats on a 40% protein diet than in the other 2 groups, and the degree of localization of the disease was better. The number of bacilli in lesions was smaller in the 40% series and the extent of pneumonia was less. Corresponding with the lesser extent of pneumonia there was less dissemination of tuberculous lesions. A characteristic

<sup>8</sup> Ratcliffe, H. L., Chapter 22 in *The Rat in Laboratory Investigation*, Griffith, J. Q., and Faris, E. J., Lippincott Company, Philadelphia, 1942.



feature of the long-lived animals was a progressive change in the cellular type of reaction from an early infiltration of mononuclears with little cytoplasm to a stage of consolidation with epithelioid cells and finally to a "regressive" type of mononuclear reaction in

which lesions decreased in size, epithelioid cells were few and mononuclears with little cytoplasm predominated.

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### 17116. Ante-Mortem Failure of the Aural Microphonic in the Guinea Pig.\*

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(Introduced by H. Lester White.)

From the Central Institute for the Deaf and Department of Otolaryngology, Washington University, St. Louis, Mo.

In the course of an extended study of the electrical response of the cochlea of the guinea pig to pure tones and to clicks we have collected incidental observations on the conditions of survival of these responses. Since these conditions are pertinent to any theory of the origin of the aural microphonic or "cochlear response" we present them herewith. Our report concerns only the aural microphonic, which apparently is closely related to the physical movements of the basilar membrane or some closely adjacent structure, and not the action potentials of the auditory nerve (or spiral ganglion) which are usually recorded with the microphonic. In general the action potentials fail equally with or before the microphonic. We have never yet observed the action potentials under conditions where the aural microphonic could not also be elicited, although sometimes a greater intensity of stimulus is necessary to make the microphonic visible on the oscilloscope.

Our sound-generating system and our pick-up amplifiers and oscilloscopes follow conventional design and will be described in detail elsewhere. We make electrical contact with the cochlea through a tiny wick applied to the round window and through fine copper or silver wires passing through or fitted like

a cork into small holes drilled through the bony shell of the cochlea. A reference electrode is connected to the edge of the wound in the neck.

Mere placement of an electrode on the round window membrane does not necessarily cause a reduction of the aural microphonic over a period of many hours or even 2 or 3 days. Neither does the anaesthetic *per se* (dial in urethane), provided the dose is light enough to allow reflex muscular movements and circulation and respiration remain adequate. Fall in body temperature sometimes seems to cause a reversible reduction of the microphonic, but no more than can reasonably be explained by the associated fall in metabolic rate and changes in circulation and respiration. Such effects of temperature have been more prominent in our experiments on cats than in guinea pigs.

It is well known that the aural microphonic falls off sharply when the animal dies, but that it persists for many hours after death at a few per cent of its original strength.<sup>1</sup> In the guinea pig, when respiration gradually fails from too much anesthetic, the microphonic falls to this low level *before* the heart stops. The failure is partly reversible. If the animal takes a few gasping breaths the microphonic may double its voltage (and action potentials return) as the heart beats faster and more regu-

\* This work was carried out under Contract N6onr-272 between the Office of Naval Research and Central Institute for the Deaf.

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<sup>1</sup> Wever, E. G., Bray, C. W., and Lawrence, M., *Ann. Otol., Rhinol., and Laryngol.*, 1941, 50, 317.

TABLE I.  
Changes in Threshold (Decibels) for 20 Microvolt Response at Round Window (1000 Cycles)  
following Deliberate Injury.

Exp. No.	Injury	Time after injury			
		1 hr	2 hr	3 hr	21 hr
60	Turn 3; all 3 sealae	+ 4	- 3	—	- 7
63	" 3; " " "	+14	+13	—	—
66	" 2; large hole in scala media	0	—	—	—
67	" 3; all 3 sealae, hole enters the modiolus	- 2	—	—	—
68	" 3; amputation	+ 2	+ 4	+12	—
70	" 3; amputation	- 4	- 4	—	—

Positive values indicate rise in threshold, negative a fall.

See Fig. 1A for Experiment No. 60, 1B for No. 67.

larly. It is our clear impression that there are two mechanisms underlying the microphonic, one of which is quite sensitive to lack of oxygen and the other sufficiently hardy to persist (at a low level) for hours after death.

Frequently, but not always, as respiration and circulation fail and as the microphonic approaches the postmortem level, the microphonic undergoes *half-wave rectification*. The phase of the microphonic in which the scala media and scala vestibuli are relatively more positive and the scala tympani more negative is depressed much more than the opposite half of the pattern. The phase which is most depressed corresponds to *condensation* in the external canal. This effect is most dramatic on the oscilloscope when clicks are used as stimuli, because the quiet base-line before the click gives an obvious reference level; but rectification can be clearly seen with pure tones also. The rectification is almost instantly reversible if the animal takes a few good breaths. At this stage rather strong stimuli, 30 or 40 db stronger than are necessary for a fresh preparation, are required to obtain clear responses and the maximum responses are rarely more than 10 microvolts. Action potentials always fail when or before rectification begins.

There are several sufficient conditions for failure of the microphonic, including lack of oxygen, overstimulation, certain chemicals applied to the round window (including the orthophosphoric acid we use in mixing the dental cement that attaches our electrode wires to the edge of the surgical opening into the bulla.) Gross injury, such as rupture of the round window membrane with outpouring of perilymph and perhaps entrance of air bub-

bles, also abolishes all responses.

Milder surgical injury associated with drilling a hole 300  $\mu$  in diameter and deliberately rupturing Reissner's membrane and the basilar membrane does not necessarily cause any deterioration of electric response over several hours except for a narrow band of frequencies associated with the actual site of injury. (See Table and Figure). In two experiments complete amputation of the 3d and 4th turns caused very little change in two hours of the stimulus necessary to yield at the round window a 20 microvolt response to medium and high tones. Small holes (60  $\mu$ ) at the apex without rupture of the endosteum seem quite innocuous. We have not detected any effects that we must attribute to a moderate "escape of endolymph" or to "serous labyrinthitis."

Two general conditions are important, and they may reduce ultimately to one. They are 1) a general factor associated with progressive deterioration of the preparation in a long experiment. The electrical responses may gradually fall and thresholds rise. High tone responses are usually more depressed than low. 2) Local oxygen supply, which may be reduced by failure of circulation or by inadequate respiration. For example, the responses fail within a few seconds of cessation of respiration under pure nitrous oxide and return quite or nearly to normal promptly when breathing is resumed. The general factor suggested above may be nothing more than cumulative irreversible or slowly reversible effects of prolonged partial anoxia.

The stability of the responses in the presence of holes drilled into the cochlea opens the way for more intimate approach to the

feature of the long-lived animals was a progressive change in the cellular type of reaction from an early infiltration of mononuclears with little cytoplasm to a stage of consolidation with epithelioid cells and finally to a "regressive" type of mononuclear reaction in

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TABLE I.  
Comparison of Inhibition Titer of Two Samples of Rabbit Serum and Egg White on Hemagglutination by PR8 and Lee Strains.

Strain	Treatment	Inhibition titer* of			
		Rabbit serum No. 1	Rabbit serum No. 2	Egg white No. 1	Egg white No. 2
PR8	unheated	<10	<10	10	<10
	30' 56°	<10	<10	40	<10
Lee	unheated	40	40	10	<10
	30' 56°	160	160	2560	640

\* Numbers give highest dilutions of inhibitor which are effective when added to virus.

virus, it was suggested that<sup>12,14</sup> the inhibition phenomenon could be explained on the basis of an enzymatic reaction as had previously been proposed<sup>1</sup> for the action of virus on red cells. According to the enzymatic theory, heating inactivates an enzyme of the virus which in the fresh state is able to destroy the inhibitor. The question arises whether all inhibitors are of the same chemical nature or whether different substances are involved. In terms of the enzymatic theory a plurality of inhibitors might also necessitate the assumption of different enzymatic factors in the virus. To obtain some evidence which could be used for answering this question, the behavior of different influenza strains toward the inhibitors contained in normal rabbit serum and egg white was compared.

*Materials and methods. Virus Preparation.* Allantoic fluid from eggs infected with the different strains of virus was used either unheated or after heating for 30 minutes at 56° or 61°C, during the first 14 days after harvesting. The following strains were examined: Swine, Oti, PR8, Weiss, Marton, Baum, FMI, Lee and Warner. The passage history of these strains is given in Table II.

*Inhibitors.* (a) Rabbit serum. Blood was drawn from normal, fully grown rabbits by cardiac puncture. After clotting and centrifugation, the serum (NRS) was pipetted off and heated for 30 minutes at 56°C.

(b) Egg white. The egg white (EW) of several eggs was separated from the yolk and filtered twice through a single layer of gauze.

All fluids were stored at +4°C in rubber stoppered test tubes.

*Inhibition tests.* A titration of virus using Salk's method<sup>15</sup> was made before every test. A virus concentration equal to eight times the highest hemagglutinating dilution was used in the inhibition test. Two-fold dilutions of inhibitor in saline starting with a 10-fold dilution were prepared in 0.25 cc amounts, and to all dilutions 0.25 cc of the indicated dilution of virus was added. After thorough mixing the tubes stood at room temperature for 30 minutes. Then 0.5 cc of 0.25 per cent suspension of chicken blood cells was added. Results were read after 75 minutes. A parallel titration of the virus dilution used in the inhibition test was made in every experiment to insure that the right amount of virus had been used.

*Results. Comparison of different batches of inhibitors.* In a preliminary experiment the inhibiting action of 2 different batches of rabbit serum and egg white on the hemagglutination of PR8 and Lee strains was examined. It can be seen in Table I that the 2 rabbit sera had the same inhibition titer, but the 2 batches of egg white gave different results. The inhibition titers with Batch No. 1 of egg white were higher than with Batch No. 2. In comparing the effect of an inhibitor on different influenza strains, it is, therefore, important to use the same sample of inhibitor for the whole series. The experiments reported below were all done with the same samples of rabbit serum and egg white.

*Susceptibility of unheated virus to NRS and EW.* Table II shows that the examined

<sup>12</sup> Hirst, G. K., *J. Exp. Med.*, 1948, **87**, 301, 315.

<sup>13</sup> Francis, T., Jr., *J. Exp. Med.*, 1947, **85**, 1.

<sup>14</sup> Burnet, F. M., *Lancet*, 1948, **254**, 7.

<sup>15</sup> Salk, J. E., *J. Immunol.*, 1944, **49**, 87.

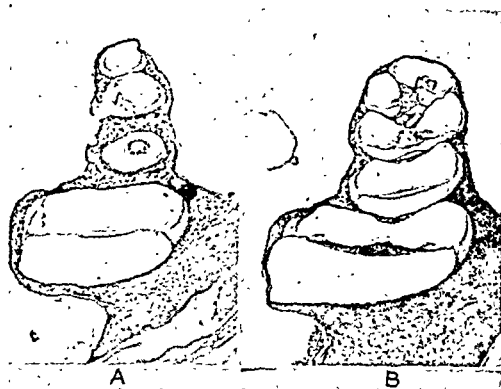


FIG. 1.

A. Exp. 60. Large hole and deliberate injury in Turn 3. Reissner's membrane and basilar membrane torn. Beneath the large hole is the outer end of a small hole that entered Turn 2, scala media.

B. Exp. 67. Hole entering modiolus in Turn 3. Axis of hole is perpendicular to plane of section.

nervous structures in labyrinth and modiolus.

*Summary.* The aural microphonic of guinea

pigs was recorded from the round window and from copper wires applied to or passing through tiny holes drilled through the bony shell of the cochlea. When the guinea pig's respiration fails gradually, the microphonic falls to a few percent of its original strength before the heart stops. The failure is partly reversible if respiration improves.

Frequently near the postmortem level the microphonic undergoes partial or complete half-wave rectification. The phase corresponding to condensation in the external canal is more depressed than the phase corresponding to rarefaction. This effect also is temporarily reversible.

Extensive surgical injury including amputation of the two apical turns may cause little or no change in the microphonic (1000 cycles per second) at the round window over several hours.

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### 17117. Differences in Hemagglutination by Strains of Influenza Virus in Presence of Egg White and Normal Serum.\*

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Substances of various origin have been shown<sup>1-10</sup> to inhibit the hemagglutination re-

action of influenza virus. Evidence has also been presented<sup>5,11,12</sup> suggesting that some of these inhibitors are identical with, or closely related to, the receptor substance of red cells. When it was shown<sup>13</sup> that serum inhibits hemagglutination by heated virus to a much higher degree than hemagglutination by fresh

\* This investigation was conducted under the auspices of the Commission on Influenza, Army Epidemiology Board, Office of the Surgeon General, U. S. Army, Washington, D.C.

† Aided by a fellowship from the Dazian Foundation for Medical Research.

1 Hirst, G. K., *J. Exp. Med.*, 1942, **75**, 49.

2 Hirst, G. K., *J. Exp. Med.*, 1943, **78**, 99.

3 Burnet, F. M., *Austral. J. Sc.*, 1947, **10**, 21.

4 Green, R. H., and Woolley, D. W., *J. Exp. Med.*, 1947, **86**, 55.

5 Friedewald, W. F., Miller, E. S., and Whitley, L. R., *J. Exp. Med.*, 1947, **86**, 65.

6 Lanni, F., and Beard, J. W., *Proc. Soc. Exp. Biol. and Med.*, 1948, **68**, 312, 442.

7 Bovarnick, M., and deBurgh, P. M., *J. Exp. Med.*, 1948, **87**, 1.

8 Ginsberg, H. S., Goebel, W. F., and Horsfall, F. L., Jr., *J. Exp. Med.*, 1948, **87**, 411.

9 Hardy, P. H., Jr., and Horsfall, F. L., Jr., *J. Exp. Med.*, 1948, **88**, 63.

10 Francis, T., Jr., and Minuse, E., *Proc. Soc. Exp. Biol. and Med.*, 1948, **69**, 291.

11 Burnet, F. M., McCrea, J. F., and Anderson, S. G., *Nature*, 1947, **160**, 404.

With other strains the inhibition of hemagglutination by NRS could be raised to titers similar to those attained by EW if a temperature of 61°C was used for the inactivation of the virus preparations. This is shown in Table III for the PR8, Weiss, Oti and Lee strains. NRS in dilutions of 640-1280 inhibited hemagglutination by heated virus, and thus came close to the values obtained with EW. In the case of the swine influenza strain it was impossible to raise its susceptibility toward NRS as a temperature of 56°C was ineffective and a temperature of 58°C destroyed the hemagglutinating ability of this strain.

*Discussion.* The observation that an influenza strain may be highly susceptible to one inhibitor and only slightly susceptible to another and the fact that heating will alter the susceptibility of a strain to one inhibitor without changing measurably its susceptibility toward the other is considered evidence for a qualitative difference in the inhibitors involved. If this conclusion is discussed in terms of the enzymatic theory it would have to be assumed that the effect of virus on the two inhibitors is caused by two different enzymatic factors which can exist independently and which can be differentiated by their resistance to heat. The virus factor active against egg white inhibitor is generally more heat labile than the factor against rabbit serum. It is completely or partially destroyed in all strains by a temperature of 56°C whereas, a

higher temperature is necessary to inactivate the factor acting against rabbit serum. The independence of the two factors is most clearly demonstrated in the case of the swine influenza strain in which heating to 56°C destroys completely the activity against egg white without measurably altering its activity against serum inhibitor. It is possible that the assumption of still other enzymatic factors for other inhibitors might become necessary, if the enzymatic theory is to be accepted. It seems probable that just as the antigenic composition of strains differs so also will their capacity to react with different chemical substrates be found to vary.

*Summary.* The hemagglutinating activity of some strains of influenza virus is more susceptible to egg white inhibitor while that of others is more susceptible to inhibition by rabbit serum. Heating to 56°C makes all strains susceptible to inhibition by egg white. When tested with 4 different strains a higher temperature was required to make them as susceptible to inhibition by normal rabbit serum. It is concluded from these results that the inhibitors are not identical. The results are discussed in terms of the enzymatic theory according to which two different enzymatic factors in the virus active against rabbit serum and egg white would have to be assumed.

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### 17118. Effect of Tagathen\* on Histamine-Induced Gastric Lesions in Guinea Pigs.

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The use of synthetic antihistaminics to prevent or diminish the incidence or severity of histamine-induced ulcers in animals has been reported in less than a half dozen papers. Most of these were concerned with the failure of benadryl\* (B-dimethylaminoethylbenzhydryl ether HCl) to prevent experimental

ulcer formation.<sup>1-4</sup> In all of these reports the "histamine in beeswax" method of Wangenstein and coworkers<sup>5</sup> was used. In addition

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<sup>1</sup> Crane, J. T., Lindsay, S., and Dailey, M. E., *Am. J. Dig. Diseases*, 1947, 14, 56.

<sup>2</sup> Friesen, S. R., Baronofsky, I. D., and Wangenstein, O. H., *Proc. Soc. Exp. Biol. and Med.*, 1946, 63, 23.

\* Brand of antihistaminic drug.

TABLE II.  
Inhibiting Action of Rabbit's Serum and Egg White on Hemagglutination of Nine Different Strains of Influenza Virus.

Strain	Passage	Treatment	Inhibition titer† of rabbit serum	Inhibition titer of egg white
Swine	1976 M38 E25*	—	<10	10
Oti	M179 E37	30' 56°	<10	2560
		—	10	40
PR8	198 M593 E86	30' 56°	40	5120
		—	<10	20
Weiss	F3 M32 E41	30' 56°	<10	80
		—	10	40
Marton	E10	30' 56°	20	5120
		—	160	40
Baum	F19 M10 E15	30' 56°	320	5120
		—	40	160
FMI	Lilly M37 E17	30' 56°	1280	5120
		—	40	80
Lee	F8 M137 E122	30' 56°	2560	5120
		—	40	10
Warner	E6 M17 E2	30' 56°	160	2560
		—	640	20
		30' 56°	2560	5120

\* Number after F = number of ferret passages; number after M = number of mouse passages; number after E = number of egg passages.

† Numbers give highest dilutions of inhibitor which are effective when added to virus.

TABLE III.  
Inhibition of Rabbit Serum on Influenza Strains Heated to 56°C and 61°C for 30 Min.

Strain	Treatment	Dilution of rabbit serum									
		10	20	40	80	160	320	640	1280	2560	5120
PR8	56°	+	+	+	+	+	+	+	+	+	+
	61°	—	—	—	—	—	—	—	—	—	—
Weiss	56°	—	—	+	+	+	+	+	+	+	+
	61°	—	—	—	—	—	—	—	—	—	—
Oti	56°	—	—	—	+	+	+	+	+	+	+
	61°	—	—	—	—	—	—	—	—	—	—
Lee	56°	—	—	—	—	—	+	+	+	+	+
	61°	—	—	—	—	—	—	—	+	+	+

+ = hemagglutination.

— = no hemagglutination.

strains differ in their behavior toward the 2 inhibitors. Six strains are more susceptible to inhibition by EW, three strains are more strongly inhibited by NRS. The greatest difference in behavior toward the 2 inhibitors was observed in the case of the Warner strain which was inhibited in a 32-fold higher dilution of NRS than of EW.

*Susceptibility of heated virus to NRS and EW.* Heating for 30 minutes at 56°C made all strains except PR8 highly susceptible to the inhibiting action of EW. Inhibition titers of 2560 to 5120 were obtained. The susceptibility to inhibitor after heating of the virus was one hundred to several hundred fold greater with all strains except PR8 with which the

rise was only 4-fold. The effect of heating virus preparations to 56°C on the susceptibility to inhibition by serum was different. In 5 strains the rise in susceptibility was only 2 to 4-fold and in the case of PR8 and swine influenza strain 1976 this temperature had no measurable effect on the susceptibility to NRS. Especially striking was the behavior of the swine influenza strain. Its susceptibility to EW rose several hundred times, while its susceptibility to NRS was not changed. A similar observation with the same virus has already been made by Lanni and Beard." With two strains the rise in titer of NRS was 30 to 60-fold and thus approached the values observed with EW.

TABLE I.  
Influence of Tagathen on Production of Histamine-Induced Ulcers in Guinea Pigs.

Treatment*	No. animals	Animals showing gastric pathology	
		No.	%
Kale†	11	0	0
Kale† + histamine‡	45	44	98
Histamine‡	25	23	92
No Vit. C + histamine‡	7	5	71
20 mg Vit. C + histamine‡	6	5	83
4 " tagathen§	6	0	0
4 " " + histamine‡	6	0	0
6 " " " " "	6	1	17
0.1 " " " " " + kale†	6	6	100
0.5 " " " " " " "	6	6	100
1 " " " " " " "	18	10	56
2 " " " " " " "	12	7	58
4 " " " " " " "	50	24	48
6 " " " " " " "	19	9	47
8 " " " " " " "	6	1	17
12 " " " " " " "	5	1	20

\* All groups received basal diet as given except as noted.

† Kale—fed *ad libitum*.

‡ Histamine diphosphate (0.6 mg) in distilled water injected i.m./pig 2×/day.

§ Tagathen in distilled water orally/pig 2×/day.

histaminic to prevent histamine-induced ulcers. It would appear that such a reduction in ulcer production in so large a group of animals clearly demonstrates the potent anti-ulcer properties of this compound.

In addition to the above reported experiments we have made preliminary investigations on the influence of desiccated thyroid, thiouracil, dried liver cake, 15-unit liver extract, insulin, thyroxine, tannic acid, 2,4-dinitrophenol and casein digests on the incidence of histamine-provoked gastric lesions.<sup>5</sup> None of these was found to have any marked preventive or provocative activity.

*Summary.* Conditions have been estab-

lished for the production of gastric lesions in guinea pigs by injection of aqueous histamine without considerable loss of animals due to fatal bronchial spasm.

Tagathen causes a very pronounced reduction in the incidence of gastric lesions produced by this method.

In view of the favorable results obtained, it would seem that a study of the mechanism involved in the anti-ulcer effect produced by antihistaminic compounds of this type, should be made.

We wish to acknowledge the invaluable technical assistance of Everett Snedeker in this work.

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## 17119 P. Blood Flow and Oxygen Consumption of the Brain in Coarctation of the Aorta.\*

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(Introduced by Carl F. Schmidt.)<sup>‡</sup>

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Recent developments making possible meas-

\* The expenses of these studies were defrayed by a grant from the Life Insurance Medical Research Fund to the laboratory of pharmacology.

urements of the blood flow to the brain<sup>1,2</sup> and myocardium<sup>3</sup> in man<sup>4</sup> enable the clinical in-

<sup>†</sup> Dr. Crumpton is National Health Institute Fellow.



in a recent paper, the pyloric ligation method of Shay and coworkers<sup>6</sup> was used to evaluate the anti-ulcer effect of thephorin\* (2-methyl-9-phenyl-2,3,4,9 tetrahydro-1-pyridindene).<sup>7</sup>

In our initial attempts to establish an assay for the investigation of the influence of diet on histamine-induced ulcer, we tried the histamine in beeswax method. In our hands it frequently resulted in the death of a considerable number of animals due to bronchial spasm. To prevent this the animals were pretreated with tagathen\* (N,N-dimethyl-N'-(2-pyridyl)-N'-(5-chloro-2-thenyl)-ethylene-diamine citrate) or with epinephrine. Under these conditions we were able to produce only an occasional gastric lesion in a large number of animals. After numerous trials it was found that gastric lesions could be consistently produced by the intramuscular injection of 0.6 mg histamine diphosphate in an aqueous solution twice daily. The number of deaths due to bronchial spasm was practically nil. This led to the study of the influence of diet and various drugs on histamine-induced ulcer in guinea pigs.

**Method.** Hartley strain guinea pigs weighing 200-350 g were divided into groups containing equal numbers of each sex and average weight. Animals were placed in individual cages and fed the appropriate diet for 5-7 days. At the end of this period the drug supplement was administered twice daily, morning and afternoon, one hour before the intramuscular injection of 0.6 mg histamine diphosphate in distilled water. Food was removed from cages before the first histamine injection and returned 2-3 hours after the

second injection. This treatment was continued for 4½ days and all surviving animals were killed and autopsied. Animals succumbing before the end of the test were autopsied as soon as possible after death. Animals were examined for the presence or absence of gastric hemorrhages, erosions and/or perforation.

The synthetic diet used in this work has the following composition expressed in grams: Cerelese 500, Alcohol extracted casein 220, Cellulose 150, Gelatin 60, Salt mixture 50, Corn oil 20, Vitamin A 19,720 I.U., Vitamin D 2,360 I.U., Vitamin E 0.025, Vitamin K .0005, Riboflavin 0.005, Inositol 0.05, Thiamine HCl 0.003, Pyridoxine HCl 0.005, Calcium pantothenate 0.02, Nicotinamide 0.01, p-aminobenzoic acid 0.01, Choline Cl 1.0, Biotin 0.0001, Folic Acid 0.001.

The animals were given 20 mg ascorbic acid orally 3 times per week. This diet was also fed with kale *ad libitum*.

**Results.** The results are presented in Table I. A high incidence of histamine-induced lesions were obtained on both the basal diet, and the basal plus kale *ad libitum*. On the former the percentage was 92%; on the latter 98%. The omission or addition of extra ascorbic acid to the synthetic diet did not appreciably influence the effect of the histamine.

Since our earlier work indicated that tagathen administration interfered with the production of ulcers by the histamine in beeswax technic, it was decided to investigate the effect of this antihistaminic drug thoroughly. The data in Table I rather clearly demonstrate that the compound if given in sufficient dosage, will prevent a high percentage of the animals from developing gastric lesions. This is attested by the fact that out of 92 guinea pigs receiving 4 or more milligrams of this antihistaminic drug per dose, only 36 exhibited gastric lesions. A considerable number of those affected showed only minimal damage as compared to their controls. At higher dosages than 4 mg the percent incidence is further reduced. This, we believe to be remarkable in view of the previously reported inability of this type of anti-

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<sup>4</sup> Vallery-Radot, P., Halpern, B. N., and Martin, J., *Presse Med.*, 1947, **55**, 185.

<sup>5</sup> Hay, L. J., Varco, R. L., Code, C. F., and Wangenstein, O. H., *Surg., Gynec. and Obst.*, 1942, **75**, 170.

<sup>6</sup> Shay, H., Komarov, S. A., Fels, S. S., Merenze, D., Gruenstein, M., and Siplet, H., *Gastroenterology*, 1945, **5**, 43.

<sup>7</sup> Lehmann, G., and Stefko, P. L., *J. Lab. and Clin. Med.*, 1949, **34**, 372.

have had the opportunity to make measurements of the cerebral circulation in two patients with hypertension limited to the upper part of the body by reason of their having coarctation of the aorta. Our results indicate that the cerebral vascular resistance in these patients is lower than that found in essential hypertension.

*Methods.* Cerebral blood flow, cerebral oxygen consumption, mean femoral pressure and brachial artery auscultatory pressure measurements were made in two patients with coarctation and in forty-five with essential hypertension.<sup>2</sup> The mean pressure of the coarctation patients was calculated by using the diastolic pressure plus one third the pulse pressure as obtained by brachial artery auscultation.<sup>14</sup> The pressure in the carotid artery was assumed to be the same as the brachial artery pressure. The cerebral vascular resistance for the essential hypertension patients was calculated as previously described.<sup>2</sup>

*Results.* These are presented in Table I. Cerebral blood flow and calculated mean carotid pressure are significantly increased when compared with the mean values of the young

normal male.<sup>2</sup> These data indicate that the cerebral vascular resistance in coarctation is normal, and is not increased as in essential hypertension. Cerebral oxygen consumption is also increased with cerebral arteriovenous oxygen difference not significantly different from the normals or hypertensives.

*Discussion.* Because S.S. had different blood pressures in each arm the higher pressure (127 mm Hg) is assumed to be more representative of the pressure in the cerebral arterial circuit. These pressure values probably underestimate mean carotid artery pressure by about 5 mm Hg because brachial artery auscultation is a lateral artery pressure measurement. This does not take the coarctation cerebral vascular resistance values out of the normal range.

*Summary.* 1. Measurements of cerebral blood flow, oxygen consumption and cerebral vascular resistance were made in two patients with coarctation of the aorta. The resistance to blood flow through the brain is found to be within the normal range.

2. This is in contrast with the increased cerebral vascular resistance of essential hypertension.

<sup>14</sup> McLeod's Physiology in Modern Medicine, 8th edition, edited by Philip Bard, Chap. 21, p. 314.

investigator of the hemodynamics in coarctation of the aorta to make for the first time an accurate estimate of the pressure-flow relationship in these regions. Reports concerning the circulatory alterations in this malformation have not established decisively whether or not vascular resistance is increased uniformly throughout the body.<sup>5-10</sup> Bing suggests that there is no generalized elevation of peripheral vascular resistance.<sup>11</sup> This preliminary report is to present evidence showing that cerebral blood flow and oxygen consumption in two patients with coarctation are significantly increased above normal.

Studies in this laboratory over the past four years have indicated that there is increased resistance to blood flow through the brain in arterial hypertension of unknown cause, this region sharing in equal measure the generalized increase in vascular tone which is associated with essential hypertension.<sup>12,13</sup> We

† The authors wish to acknowledge the assistance of Drs. C. F. Schmidt, Seymour S. Kety and William A. Jeffers in making these studies.

<sup>1</sup> Kety, S. S., and Schmidt, C. F., *Am. J. Physiol.*, 1945, **143**, 53.

<sup>2</sup> Kety, S. S., and Schmidt, C. F., *J. Clin. Invest.*, 1948, **27**, 476.

<sup>3</sup> Ekenhoff, J. E., Hafkenschiel, J. H., Harmel, M. H., Goodale, W. T., Lubin, M., Bing, R. J., and Kety, S. S., *Am. J. Physiol.*, 1948, **152**, 356.

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<sup>5</sup> Blumgart, H. L., Lawrence, J. S., and Ernestine, A. C., *Arch. Int. Med.*, 1931, **47**, 806.

<sup>6</sup> Lewis, T., *Heart*, 1933, **16**, 205.

<sup>7</sup> Pickering, G. W., *Clinical Science*, 1935-1936, **2**, 209.

<sup>8</sup> Prinzmetal, M., and Wilson, C., *J. Clin. Invest.*, 1936, **15**, 63.

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<sup>10</sup> Ryttand, D. W., *J. Clin. Invest.*, 1938, **17**, 391.

<sup>11</sup> Bing, R. J., Handelsman, J. C., Campbell, J. A., Griswold, H. E., and Blalock, Alfred, *Ann. Surg.*, 1948, **128**, 803.

<sup>12</sup> Kety, S. S., and Schmidt, C. F., *Am. J. Med. Sciences*, 1946, **212**, 124.

<sup>13</sup> Kety, S. S., Hafkenschiel, J. H., Jeffers, W. A., Leopold, Irving H., and Shenkin, Henry A., *J. Clin. Invest.*, 1948, **27**, 511.

TABLE I.  
Cerebral Circulatory Data with Normal Circulation,<sup>2</sup> Essential Hypertension, and Coarctation of the Aorta.

Group	Blood pressure, mm. Hg.		Cerebral blood flow, cc/100 g/min.		Cerebral vascular resistance, M.A.P./C.B.F. O <sub>2</sub> diff. vol. %		Cerebral oxygen consumption, % cc/100 g/min.	
	Indirect		Direct		Femoral mean		arteriovenous	
	Brachial		Left		Mean		diff.	
	Obs.	Mean	Obs.	Mean	Obs.	Mean	vol. %	cc/100 g/min.
Young normal ♂ (24 observations, 14 subjects)	107/69	82	107/69	82	86		6.3	3.3
Coarctation group, mean							6.0	*4.6
S.S. 18 WM	130/105	113	180/100	127	107		6.0	5.2
R.F. 20 "	150/80	103			84		5.9	4.0
Essential hypertension (45 observations, 45 patients)					*156		6.5	3.2

\* Denotes statistically significant difference from normals.

† This is the average value of mean brachial artery pressure for the two coarctation patients where the higher brachial artery pressure of S.S. (127) was chosen as the one more closely approximating the value of mean carotid artery pressure.





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### SECTION MEETINGS

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University of Wisconsin	April 28, 1949

### 17120. Proteolytic Enzyme Inhibitors of Human Serum in Health and Disease.\*†

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The antiproteolytic power of blood serum has been described in the reports of Flexner

*et al.*,<sup>1</sup> Sure,<sup>2</sup> Weil and Russell<sup>3</sup> and Clark *et al.*<sup>4</sup> Following purification of several proteolytic enzymes it became possible to determine the probable nature of this enzyme antagonism. Crystalline pepsin inhibitor isolated by Herriott<sup>5</sup> and trypsin inhibitor by

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<sup>1</sup> Flexner, L. B., Berkson, J., Winters, H., and Wolman, I., *Proc. Soc. Exp. Biol. and Med.*, 1929, **26**, 592.

<sup>2</sup> Sure, B., *Biochem. J.*, 1935, **29**, 1508.

<sup>3</sup> Weil, L., and Russell, M. A., *J. Biol. Chem.*, 1938, **126**, 245.

<sup>4</sup> Clark, D. G. C., Clifton, E. E., and Newton, B. L., *Proc. Soc. Exp. Biol. and Med.*, 1948, **69**, 276.

<sup>5</sup> Herriott, R. M., *J. Gen. Physiol.*, 1941, **24**, 325.



TABLE I.  
Occurrence of Abnormal Levels of Chymotrypsin and Rennin Inhibitors in Various Groups of Patients.

	No. of cases	Abnormal chymo and/or rennin	
		No.	%
1. Pregnancy (all stages)	103	88	85.3
2. Malignancy (entire group)	220	130	59.0
" (active, untreated)	133	118	88.7
" (treated)	87	12	13.7
3. Allergy	33	9	27.2
4. Acute infections, active phase (bacterial and virus)	176	45	25.5
5. Benign breast lesions	33	8	24.2
6. Cardiovascular (entire group)	101	20	19.8
" (decompensation)	23	12	52.1
" (hypertension)	22	8	36.3
7. Chronic infections (tuberculosis, syphilis, coccidiomycosis, bronchiectasis, etc.)	140	26	18.5
8. Psychoneuroses and psychoses	55	10	18.1
9. Traumatic damage and surgical procedures (within 1 week)	280	45	16.0
10. Peptic ulcer, ileitis, colitis	83	6	7.2
11. Metabolic (diabetes, cirrhosis, nephritis, arthritis, hyperthyroidism)	137	7	5.1
12. Benign tumors (polyps, papilloma, fibromyoma, fibroma, lipoma, adenoma)	75	4	5.3
13. Unclassified	346	13	3.7
14. Healthy subjects (including routine pre-operative hernia, hemorrhoids, varicose veins, etc.)	218	0	0.0
Total	2000	411	20.5

TABLE II.  
Pathological and Physiological States Influencing Chymotrypsin and Rennin Inhibitors.

A. Pathology influencing both chymotrypsin and rennin inhibitors with equal frequency.	
1. Pregnancy.	
2. Malignancy	
3. Trauma and surgery	
4. Cardiac decompensation	
5. Chronic infections	
B. Pathology influencing chymotrypsin inhibitor predominantly.	
1. Acute infections (bacterial and virus)	24% of cases
C. Pathology influencing rennin inhibitor predominantly.	
1. Hypertension	31.8%
2. Benign breast lesions	24.2
3. Allergy	21.2
4. Psychoneuroses	18.1
5. Fractures (healing stage)	17.5

exact condition of the patient at the time the inhibitor determination was made. Results were correlated with these facts and the conclusions are summarized in Tables I and II.

*Discussion.* From a survey of data presented, it seems probable that rennin and chymotrypsin inhibitors reflect different phases of metabolism, since the concentration of one is seen to vary independently of the other.

However, in disturbances of fluid balance, both may follow the same trend. With diminished fluid output in persistent vomiting, burns, hemorrhage, shock, high fever or congestive failure, both values frequently increase together. That the full explanation may be more complex is suggested by the fact that most of the above conditions are characterized by increased excretion of adrenal cortical ster-



Kunitz and Northrop<sup>6</sup> have been shown to be highly specific, reacting to form inert dissociable complexes with the respective proteinase. As a sensitive means of studying protein metabolism in various pathological states, an investigation of the role of certain of the circulating proteolytic enzyme inhibitors appears to offer a new approach of considerable promise. This paper presents a method for quantitative estimation of both rennin and chymotrypsin inhibitors of serum, together with results obtained in a survey of 2000 patients.

*Materials and methods.* The method is based on the milk coagulating property of both chymotrypsin and rennin. Determinations are made on fresh serum or serum stored at ice-box temperature not over 24 hours. Fasting blood is not required. 1. *Enzyme Standardization.* (a) *Chymotrypsin.* Two to 3 mg crystalline chymotrypsin (supplied through courtesy of Spicer-Gerhart Company, Pasadena, California) are dissolved in 10 ml of distilled water. 0.2 ml of solution is pipetted into two 13 x 120 mm tubes containing 2.0 ml of fresh homogenized milk (pH 6.6) which is at ice-box temperature. A stop watch is started, contents of tubes mixed quickly by rotation, and placed immediately in a water bath at  $37^{\circ} \pm 0.1^{\circ}$ . If coagulation has not occurred in 9 minutes tubes are shaken gently every 15 seconds. The time at which the first granular precipitation of calcium paracaseinate appears in the film on the wall of the tube is the end point. Dilution of enzyme solution is continued until the end point occurs at 10 minutes 45 seconds to 10 minutes 50 seconds. This precise time is selected to provide reasonable stability of the enzyme as well as optimum conditions for measurement of inhibitor. At 10 minutes, variation in inhibitor levels is barely detectable, and over 11 minutes, inhibition is too great for accurate measurement and the enzyme is unstable. It is essential that the enzyme activity fall exactly within the 5 second time limitation. The enzyme will retain this activity for about 3 hours. Stability is not improved by buffer-

ing. No variation attributable to natural homogenized milk as a substrate has been encountered in over a year of continuous use of the method. Powdered and condensed varieties are unsuited to accurate detection of the end point. Both milk and enzyme are kept in the ice-box except when actually in use.

(b) *Rennin.* One rennet enzyme tablet ("Junket" brand, Chr. Hansen's Laboratory Inc., Little Falls, N. Y.) is dissolved in 80 ml of distilled water, and filtered after 10 minutes. Activity is adjusted as for chymotrypsin to an end point of 10' 55" to 11' 0". The difference of 10 seconds between the two enzymes allows measurement of inhibitors over a comparable range. 2. *Determination of Inhibitor.* (a) *Chymotrypsin inhibitor.* Two control tubes and 6 others representing 3 duplicate serum determinations are set up together. Dilute serum 1:10 with distilled water and pipette 0.04 ml into each tube. 2 ml of milk is added and the contents mixed. Finally, 0.2 ml of standardized chymotrypsin solution is added quickly and accurately, a stop watch started, the eight tubes thoroughly mixed in 20 seconds or less, and placed together in the water bath. At 10' 45" when controls reach the end point the watch is reset. Units of inhibitor are the number of additional minutes required for the serum-containing tubes to reach the end point. Results must agree within 30 seconds. Failure to reach the end point 30 minutes beyond the control is equivalent to complete inactivation of enzyme and is therefore the maximum reading. Time units are not related arithmetically to actual units but this correction is not applied. (b) *Rennin inhibitor.* Proceed as above except that 0.05 ml of undiluted serum is required in this reaction and the maximum value obtainable is 50 units. 3. *Interpretation.* (a) *Chymotrypsin inhibitor:* normal range 5-10 units; 13-30 units abnormal. (b) *Rennin inhibitor:* normal ranges 5-10 (70%) and 20-50 units (30%); 13-18 units abnormal.

*Results.* In this preliminary survey, 2000 random blood specimens representing all available types of pathology were studied. Six to 8 weeks later, pertinent clinical and laboratory findings that had been collected on each case were assembled with special reference to the

<sup>6</sup> Kunitz, M., and Northrop, J. H., *J. Gen. Physiol.*, 1936, 19, 991.

TABLE I.  
Comparison of Total Body Water in Rabbits Determined by Antipyrine and Desiccation.

Rabbit	Weight (g)	Total water				Difference in (%)
		(cc)	Antipyrine (% body wt)	(cc)	Desiccation (% body wt)	
1	1782	1330	74.6	1350	75.8	-1.2
2	1846	1270	68.8	1285	69.6	-0.8
3	660	501	75.8	481	72.9	+2.9
4	1172	912	77.8	902	77.0	+0.8

TABLE II.  
Antipyrine in Tissues of Rabbit 50 Minutes After Intravenous Injection.

Tissue	Water (%)	Antipyrine in wet tissue (γ/g)	Antipyrine in tissue water (γ/ml)	Tissue water antipyrine
				Plasma water antipyrine
Plasma	92.0	39.6	43.1	1.00
Heart	73.7	30.5	41.3	.96
Liver	72.0	33.2	46.1	1.08
Muscle	73.0	31.2	42.8	.99
Kidney	78.6	33.1	42.1	.97
Lung	71.6	31.1	43.5	1.01

was instantaneous and if none of the substance had been metabolized) is calculated by plotting the plasma levels on semilogarithmic paper as a function of time and extrapolating the straight portion of the time concentration curve (50, 75 and 100 minutes) to the time of injection. The value for total body water is obtained by dividing the amount administered by the plasma water level at zero time.

$$\text{Body water (liters)} = \frac{\text{Amt of drug injected (mg)}^1}{\text{Plasma water level (mg/L)}}$$

After the last blood sample had been drawn, the animals were weighed and sacrificed. The total animals were ground, and the final mass was then spread over large evaporating dishes and placed in a vacuum desiccator at a temperature of 85°C for a period of 5-7 days until constant weight had been achieved.

Since the rabbit, unlike a human, degrades antipyrine at a rapid rate (approximately 40% an hour), it appeared possible that the falling blood level indicated plasma degradation and not the concentration of antipyrine in the entire body. Accordingly, the distribution of antipyrine in the tissues of a fifth rabbit at the end of 50 minutes was determined. The water content of the tissues was determined

by drying to constant weight at 100°C. The concentration of antipyrine in the tissues was measured and then calculated in terms of tissue water. The antipyrine is estimated directly in the plasma filtrate after deproteinization with zinc hydroxide. Sodium nitrite is added and the resulting 4-nitroso antipyrine measured in a spectrophotometer at 350 mμ.<sup>2</sup>

**Results.** Comparison of the values obtained for total body water by antipyrine method and desiccation indicates close agreement between the two methods, thus confirming that antipyrine can be used as a measure of total body water (Table I).

The data of Table II demonstrate that the ratio of concentration of antipyrine in the tissues to that of plasma is one. Thus, the plasma concentration reflects the level of antipyrine in the entire body.

**Summary.** The close agreement obtained for the values of total body water by the use of the antipyrine method and desiccation, indicates the validity of this procedure for the purpose of measuring total body water.

<sup>2</sup> Brodie, B., Axelrod, J., Soberman, R. J., and Levy, B., *J. Biol. Chem.*, 1949, **179**, 25.

oids having a profound influence on protein metabolism. Factors which influence the rennin inhibitor predominantly further implicate the steroid hormones. The association of estrogens with certain benign breast hyperplasias is well known and the significance of stimulated estrogenic or androgenic activity in healing fractures has been demonstrated by Albright and co-workers.<sup>7</sup> Rennin inhibitor may increase also in many of the psychoses which have been shown by Pincus and Elmadjian<sup>8</sup> to exhibit diminished adrenal response to stress. The unexpected finding that a high percentage of the hypertensive and allergic groups have abnormal rennin inhibitor levels is of interest but difficult to interpret. Studies to determine the direct effects of steroid hormone administration on the proteolytic enzyme inhibitors are being undertaken.

Pregnancy is usually accompanied by a pronounced elevation of both inhibitors in the mother's circulation. In contrast, the cord blood of the newborn is high only in rennin inhibitor, while the other is very low.

A study of malignancy by this method re-

<sup>7</sup> Albright, F., Bloomberg, E., and Smith, P. H., *Trans. Assn. Am. Phys.*, 1940, 55, 298.

<sup>8</sup> Pincus, G., and Elmadjian, F., *J. Clin. Endocrinol.*, 1946, 6, 285.

veals a characteristic but fluctuating picture, the host doing well clinically when the rennin inhibitor only is elevated, as occurs early, or at intervals in the course of the disease, but failing rapidly when the chymotrypsin inhibitor rises very high and rennin inhibitor drops to low levels (unpublished data). Obviously, it would be impossible to diagnose malignancy by these methods since an increase in inhibitor of either proteolytic enzyme is not specific for cancer. The inhibitor concentrations are profoundly influenced by radiation therapy and surgical excision, and thus a low percentage of abnormal inhibitor levels was noted in treated cases. Repeated observations on the serum inhibitors in cases with neoplasm may prove to be of considerable prognostic value and may indicate when further treatment is required as well as the effectiveness of the treatment given. This appears to us to be the chief value of the method as applied to the cancer patient. This aspect of the problem is being extended at the present time.

*Summary.* Methods are described for the determination of chymotrypsin and rennin inhibitors in serum. The many physiological and pathological conditions which influence their concentration are discussed.

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## 17121. A Comparison of Total Body Water as Determined by Antipyrine and Desiccation in Rabbits.

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A method for determining total body water which employs the volume of uniform distribution of antipyrine, an analgesic drug, has been described recently.<sup>1</sup> Further support for the antipyrine method is presented in the present paper which records the values for total body water estimated by the antipyrine

procedure and the values obtained by actual desiccation of the experimental animals.

*Experimental.* Four rabbits were injected intravenously with 60-240 mg of antipyrine according to body weight (approximately 100 mg/kg). Blood samples were drawn from each animal at 50, 75 and 100 minutes following the administration of the drug. The zero time plasma concentration (the concentration at the time of injection if uniform distribution

<sup>1</sup> Soberman, R. J., Brodie, B., Hollander, V., Levy, B., Axelrod, J., and Steele, J. M., *J. Biol. Chem.*, 1949, 179, 31.

TABLE I.  
Experimental Hypertension. Blood pressure changes associated with stenosis of the thoracic aorta in the dog.

Days after constriction	Blood pressure in mm mercury Femoral		Blood pressure in mm mercury Carotid	
	Mean	S/D	Mean	S/D
Normal	127	165/108	126	149/110
6	45	45/45	138	161/116
16	133	151/121	158	200/137
30	157	181/135	190	233/149
40	169	184/154	197	236/162
124	176	199/167	238	292/198

S = Systolic. D = Diastolic.

TABLE II.  
The Blood Pressure Changes Associated with Experimental Coarctation of the Aorta in Dogs.

Animal No.	Normal blood pressure				Duration of aortic constriction, mo.	Last recorded blood pressure			
	Femoral		Carotid			Femoral		Carotid	
	M	S/D	M	S/D		M	S/D	M	S/D
14	118		124		14	133	144/123	158	185/130
16	128		130		5	196		222	
22	134		140		13	154	170/137	193	241/160
29	127	165/108	126	149/110	4	176	199/167	238	292/198
31	125	157/107	161	184/146	3	162	170/157	239	299/199

M = Mean pressure. S = Systolic. D = Diastolic.

pulse pressures below the constriction that persisted. This might be interpreted as evidence for renal origin of the hypertension as suggested by Kohlstaedt and Page.<sup>3</sup>

The substitution of a rigid narrow tube for a section of the aorta would alter the characteristics of the pulse waves above and below the constriction; but this would not explain

the marked elevation of the blood pressure that occurs in experimental stenosis of the aorta.

*Summary.* 1. By stenosis of the thoracic aorta in dogs, arterial hypertension can be produced above and below the stenosis.

2. This type of experimental hypertension has many of the characteristics observed in the hypertension occurring in coarctation of the aorta in man.

<sup>3</sup> Kohlstaedt, K. G., and Page, I. H., *J. Exp. Med.*, 1940, **72**, 201.

## 17122. Arterial Hypertension Produced by Experimental Stenosis of the Thoracic Aorta.

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Arterial hypertension has been produced in dogs by stenosis of the thoracic aorta. The hypertension is sustained and has many of the characteristics of that observed in coarctation of the aorta in man.

*Materials and methods.* The method of producing permanent and prolonged stenosis of the thoracic aorta in dogs, described in detail elsewhere,<sup>1</sup> consists in the substitution of a section of lucite tubing for a segment of the aorta. This tube is designed to reduce turbulence by making the bore hourglass in shape. In the dogs used in this experiment, the tube was 3.7 cm long with 3 mm as the narrowest diameter. The lumen of the aorta was reduced in the dogs in this group approximately 75 to 85%.

Blood pressure records were obtained by direct puncture of the femoral or carotid arteries and measurement of the pressure with either a mercury or Hamilton manometer. From the records obtained with the Hamilton manometer, the systolic and diastolic pressures were calculated. Mean pressures were obtained either from estimations with the planimeter of the area under the pulse waves, or read directly from the mercury manometer. All blood pressure determinations were made with the animals under nembutal anesthesia, 27 mg per kg of body weight.

*Results.* There were 11 of 16 dogs that survived the operative procedure. Six of the 11 died at intervals of 7 days to 3 weeks after operation, 4 dying because of spastic paralysis of the lower part of the body. Of the 5 animals that survived for longer than one month, 4 are still living. One dog died at the end of 4 months from unknown cause. The remainder are still living at intervals of 4 to 14 months after stenosis of the aorta.

The changes in the blood pressures in these animals has followed, in all instances, a definite pattern as demonstrated in Table I. The femoral mean pressure has shown a drop after operation that persisted in some animals for as long as 7 days; but by the 14th day the pressure returned to normal. After 2 weeks the femoral mean pressure increased above the normal. The carotid mean pressure increased by the end of the first week to levels well above normal, and by 14 days reached hypertensive levels. The mean pressures in both the femoral and carotid increased during the next 3 months. After this time there was in the 2 dogs observed for one year, a decrease in the mean pressure both above and below the constriction, but the pressure still remained in the hypertensive range. The results with 5 animals are tabulated in Table II.

Recordings of the blood pressure with the Hamilton manometer have shown a large increase in the pulse pressure above the constriction and a decrease below. The diastolic pressures both above and below the constriction have increased along with the rise in the mean pressure.

*Discussion.* The experiments described here show that severe arterial hypertension can be produced in dogs by stenosis of the thoracic aorta. The various mechanisms that contribute to the elevation of the blood pressure are not evident from these results. It has been suggested that the hypertension in coarctation of the aorta is renal in origin.<sup>2</sup> Though the blood flow to the kidneys is not recorded in these experiments, it is interesting to note that for only about 14 days was the mean pressure below the constriction lower than the normal. After this interval there was a progressive rise in the mean pressure. On the other hand, there was a diminution in the

<sup>1</sup> Sealy, W. C., and McSwain, George H., *Surgery*, in press.

<sup>2</sup> Ryland, D. A., *J. Clin. Invest.*, 1938, **17**, 391.

TABLE I.  
The Blood Volume of the Adult Rat as Determined with Red Blood Cells Doubly-labelled with  $\text{Fe}^{59}$  and  $\text{P}^{32}$ .

Rat No.*	Body wt in g	Hematocrit	Blood vol. ( $\text{Fe}^{59}$ ) cc/100 g body wt	Blood vol. ( $\text{P}^{32}$ ) cc/100 g body wt	Total red cell vol. ( $\text{P}^{32}$ ) cc/100 g body wt
1	205	45.	5.06	5.26	2.36
2	213	45.5	4.69	5.58	2.54
3	185	44	4.97	5.13	2.26
4	198	43.5	5.81	5.95	2.58
5	190	49	3.63	3.94	1.93
6	180	53	4.28	4.10	2.17
7	185	48	4.11	4.32	2.07
8	174	50	4.36	4.31	2.16
9	170	50	4.41	4.12	2.06
10	233	46	4.98	5.36	2.46
11	202	50	4.95	5.10	2.55
12	218	48	5.74	5.27	2.53
Avg	196.3	47.7	4.75	4.87	2.31

\* Rats 1-9 were males; 10-12 lactating females.

counting was 1%. The  $\text{Fe}^{59}$  was assayed by a modification of Peacock's method.<sup>11</sup> The blood samples and the diluted standard were placed in 25 cc Erlenmeyer flasks; 10 mg of carrier iron, as ferric chloride, were added to the sample which was then dried at 70°C. After drying, 3-5 cc of concentrated nitric acid were added and evaporated to dryness. The ashing was completed with concentrated sulfuric acid and 30% hydrogen peroxide. The sulfuric acid solution of the ash was transferred to a centrifuge cone and the iron precipitated with concentrated sodium hydroxide. After centrifuging, the supernatant was discarded, the precipitate was redissolved in 0.25 cc of concentrated hydrochloric acid, the precipitation and centrifugation repeated, and the supernatant again discarded. The final precipitate was dissolved in 0.25 cc concentrated hydrochloric acid. The solution was transferred to an electrodeposition cell containing 35 cc of plating solution (one part saturated oxalic acid and five parts saturated ammonium oxalate). The iron was electro-deposited at 0.8 amperes and 12 volts in an apparatus designed by Dunn.<sup>12</sup> The samples and the standard were counted on a thin end window

Geiger-Mueller counter. The probable error of counting was 1%.

**Results.** The blood volume as determined by both  $\text{Fe}^{59}$  and  $\text{P}^{32}$ , together with the total red cell volume, the hematocrit and the body weight of the Group I animals are presented in Table I. The same determinations for the Group II animals, except the  $\text{Fe}^{59}$  volumes, are recorded in Table II. The coefficient of correlation between the blood volume and hematocrit was 0.64. The standard deviation for all of the animals was not significantly different from the standard deviation for groups of litter mates. The average blood volume as determined by  $\text{P}^{32}$  for both groups, excluding the lactating females, was  $4.59 \pm 0.57$  cc/100 g body weight; the total red cell mass  $2.16 \pm 0.20$  cc/100 g body weight and the hematocrit  $45.8 \pm 2.9$  %.

**Discussion.** The values reported using vital red are 4.3 cc/100 g body weight (1937)<sup>1</sup>; 6.38 cc/100 g body weight (1933)<sup>2</sup>; 6.9 cc/100 g body weight (1928)<sup>3</sup>; and 7.4 cc/100 g body weight (1929)<sup>4</sup>; with the use of Evans blue (T-1824)-7.6 cc/100 g body weight (1944)<sup>5</sup> and 7.98/100 g body weight (1941).<sup>6</sup> The total red cell volume was reported as 2.95 cc/100 g body weight;<sup>2</sup> 3.45 cc/100 g body weight;<sup>5</sup> and 3.90 cc/100 g body weight.<sup>6</sup>

The good agreement between the values of

<sup>11</sup> Peacock, W. C., Evans, R. W., Irvine, J. W., Jr., Good, W. M., Kip, A. F., Weiss, S., and Gibson, J., II. *J. Clin. Invest.*, 1946, **25**, 605.

<sup>12</sup> Dunn, R., Donner Laboratory, Univ. of Calif.

<sup>1</sup> Standard deviation.

# 17123. The Blood Volume of the Adult Rat, as Determined by $\text{Fe}^{59}$ and $\text{P}^{32}$ Labelled Red Cells.\*

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The measurement of the blood volume of the rat by the use of plasma diluents has been reported by several investigators,<sup>1-6</sup> but there have been no reports on the use of labelled red blood cells for this purpose.

$\text{Fe}^{59}$  given enterally or parenterally has been shown by Hahn and co-workers<sup>7</sup> to be incorporated into the red blood cell as an integral part of the hemoglobin molecule. The plasma iron does not exchange with the iron incorporated into the hemoglobin.<sup>8</sup> Hevesy and Zerahn<sup>9</sup> have modified the Hahn and Hevesy<sup>10</sup> method for determining the total red cell volume by using red blood cells labelled by the incorporation *in vitro* of  $\text{P}^{32}$ . Red blood cells may be labelled by both of these methods for use in the determination of the circulating blood volume.

*Method.* The first of the 2 groups of animals used consisted of 12 adult Slonaker rats, of which 3 were lactating, the second group of 30 adult rats (the first generation of a cross-breeding of highly inbred Slonaker and Curtis Dunning rats). In each group the blood type of all animals was the same. The individuals of Group I were given doubly labelled red blood cells, those of Group II, cells labelled only with  $\text{P}^{32}$ .

A single animal was injected intraperitoneally with a solution of  $\text{Fe}^{59}$ , as ferric chloride, buffered with sodium citrate and adjusted to pH 6. After sufficient time had elapsed for the  $\text{Fe}^{59}$  level in the blood to reach a constant value, approximately 5 cc was withdrawn into a heparinized syringe by cardiac puncture, placed in a paraffined 25 cc Erlenmeyer flask and incubated with  $\text{P}^{32}$  as ( $\text{Na}_2\text{HP}^{32}\text{O}_4$ ) at 37°C for 2 hours with constant rotation. The cells were washed 3 times by adding isotonic saline solution, centrifuging and removing the supernatant fluid. Then plasma containing no  $\text{P}^{32}$  was added to the washed cells to reconstitute whole blood. Ether anesthetized animals in Group I and nembutal anesthetized animals in Group II were injected into the tail vein with 0.2 cc of this reconstituted blood. After 3 minutes approximately 1.0 cc of blood was withdrawn by cardiac puncture, 0.1 cc being used for the determination of  $\text{P}^{32}$  content, 0.5 cc for the determination of the  $\text{Fe}^{59}$  and 0.25 cc for the measurement of the hematocrit in Smith hematocrit tubes. Standards were prepared by diluting 0.2 cc of the reconstituted blood in 25 cc of distilled water.

The  $\text{P}^{32}$  was assayed by drying 0.1 cc of blood on a piece of lens paper on a thin aluminum foil, wrapping the lens paper and aluminum foil in cellophane and counting on an Eck-Krebs counter-tube. In a similar manner, 0.1 cc of the diluted standard was mounted and counted. The probable error of

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† National Cancer Institute Post Doctorate Research Fellow.

‡ A.E.C. Post Doctorate Research Fellow.

§ Institute of Experimental Biology, University of California.

<sup>1</sup> Griffith, J. Q., and Campbell, R., *Proc. Soc. Exp. Biol. and Med.*, 1937, **36**, 38.

<sup>2</sup> Orten, J. M., Underhill, F. A., Mugrage, E. R., and Lewis, R. P., *J. Biol. Chem.*, 1933, **99**, 457.

<sup>3</sup> Cartland, G. F., and Koch, F. C., *Am. J. Physiol.*, 1928, **85**, 540.

<sup>4</sup> Went, S., and Drinker, C. K., *Am. J. Physiol.*, 1929, **88**, 468.

<sup>5</sup> Metcalf, J., and Favour, O. B., *Am. J. Physiol.*, 1944, **141**, 697.

<sup>6</sup> Beckwith, J. R., and Chanutin, A., *Proc. Soc. Exp. Biol. and Med.*, 1941, **46**, 66.

<sup>7</sup> Hahn, P. F., Bale, W. F., Hettig, R. A., and Whipple, G. H., *Science*, 1940, **92**, 131.

<sup>8</sup> Hahn, P. F., Bale, W. F., Lawrence, E. O., and Whipple, G. H., *J. Exp. Med.*, 1939, **69**, 731.

<sup>9</sup> Hevesy, G., and Zerahn, K., *Acta Physiol. Scand.*, 1942, **4**, 376.

<sup>10</sup> Hahn, L., and Hevesy, G., *Acta Physiol. Scand.*, 1940, **1**, 1.

## 17124. Serum Cholinesterase in Hyperthyroidism.

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Conflicting reports have appeared in the literature concerning serum cholinesterase levels in hyperthyroidism. Antopol *et al.*,<sup>1</sup> reported an increased serum cholinesterase level in twenty-two cases of untreated hyperthyroidism and a return toward normal values in 13 cases treated by iodine or operation. Milhorat,<sup>2</sup> Butt<sup>3</sup> and Faber<sup>4</sup> reported normal values in several cases of hyperthyroidism. During preparation of this manuscript an abstract of a paper by Ambrus *et al.*<sup>5</sup> came to our attention. They recorded significant changes in the serum cholinesterase in diseases of the thyroid gland as observed clinically and experimentally. They found that thiourea derivatives lowered the high values in clinical and experimental hyperthyroidism.

It is the purpose of this communication to present our findings in normal and hyperthyroid patients.

**Methods.** The serum cholinesterase was determined in cases of euthyroidism, hyperthyroidism, and non-toxic adenoma. The diagnosis was made by clinical appraisal, laboratory findings, response to 6N-propylthiouracil and/or microscopic examination of the surgically excised thyroid glands.

Venous blood was allowed to clot, the serum separated and a few drops of chloroform added to the latter for preservation. The method of determining serum cholinesterase was a modification of Stedman's procedure<sup>6</sup> where the serum and substrate were titrated

at a temperature between 36-37°C using cresol purple as the indicator. The titration was discontinuous, and was carried out at two 10 minute intervals. The average value obtained was used as the final figure.

**Results.** The range based on determinations in 33 normal adults, was 0.30-0.52 unit with the exception of 3 values (3 cases), 0.62, 0.62, and 0.22 unit. Of this series 23 were males in whom the range was 0.22 - 0.52 unit with an average of 0.42 unit. In the 10 females the comparative values were 0.39-0.62 with an average of 0.51 unit.

In Fig. 1 a summary of the findings is presented. Of the 18 cases of hyperthyroidism that were investigated 11 came under our observation before therapy was instituted or during therapy insufficient to produce an adequate clinical response. In this group the values ranged from 0.68 to 1.45 units and all but one case was higher than 0.70 unit. In 4 of these patients only an initial cholinesterase value was obtained. The remaining seven cases in this group were followed by subse-

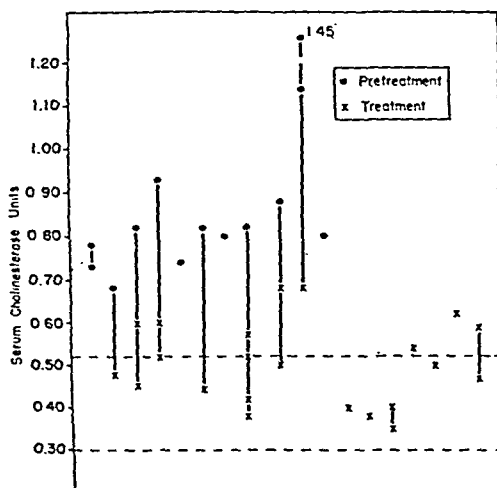


FIG. 1.

Summary of findings in 18 cases of hyperthyroidism, showing changes in serum cholinesterase with treatment.

<sup>1</sup> Antopol, W., Tuchman, L., and Schiffrin, A., *Proc. Soc. Exp. Biol. and Med.*, 1937, **36**, 46.

<sup>2</sup> Milhorat, A. T., *J. Clin. Invest.*, 1938, **17**, 649.

<sup>3</sup> Butt, H. R., Comfort, M. W., Dry, T. J., and Osterberg, A. E., *J. Lab. and Clin. Med.*, 1942, **27**, 649.

<sup>4</sup> Faber, M., *Acta Med. Scand.*, 1943, **114**, 59.

<sup>5</sup> Ambrus, K., and Ambrus, G., *Ortosok Lapja*, 1947, **3**, 865 (cited in *Excerpta Medica*, 1947, **1**, Abst. No. 473, p. 169).

<sup>6</sup> Stedman, E., Stedman, E., and Easson, L. H., *Biochem. J.*, 1932, **26**, 2056.



TABLE II.  
Blood Volume of the Adult Rat as Determined by  $\text{P}^{32}$  Labelled Red Blood Cells.

Rat No.*	Body wt in g	Hematocrit	Blood vol. ( $\text{P}^{32}$ ) cc/100 g body wt	Total red cell vol. ( $\text{P}^{32}$ ) cc/100 g body wt
13	260	49	4.27	2.08
14	240	45	4.92	2.20
16	180	46	4.77	2.22
17	165	43	5.02	2.18
18	150	45	4.17	1.88
19	230	46	4.87	2.26
20	240	47	4.34	2.04
21	230	54	3.96	2.13
22	260	47	3.92	1.85
23	230	48	4.13	1.98
24	185	50	3.46	1.78
25	225	45.5	5.50	2.31
26	232	47	5.39	2.54
27	237	47	4.33	2.10
28	190	45	4.84	2.30
29	257	44	4.82	2.17
30	225	47	5.11	2.08
31	210	44	4.24	2.04
32	205	50	4.14	2.12
33	215	50	4.41	2.51
34	235	50	4.94	2.26
35	230	44	4.52	1.99
36	295	43	4.00	1.72
37	315	46	4.23	1.95
38	320	48	4.66	2.24
39	240	46	4.50	2.07
40	200	44	4.35	1.91
41	235	43	4.81	2.07
42	245	48	4.65	2.23
43	260	45	4.99	2.25
44	240		3.79	
Avg	231.7	45.0	4.52	2.12

\* Rats 13-14, 16-17, 19-23, 25-26, 27-30, 31-32, 33-34, 35-37, 39-40, 40-42, 24 and 44 were litter mates; 16, 17, 18, 24 and 44 were females.

the blood volume as obtained by  $\text{Fe}^{59}$  and by  $\text{P}^{32}$  labelled red blood cells permits the use of the  $\text{P}^{32}$  labelled red blood cells for the determination of the blood volume of the rat. The use of  $\text{P}^{32}$  thus eliminates the comparatively lengthy and tedious processes required for the accurate assay of  $\text{Fe}^{59}$ . In addition it eliminates the need for building up in a donor animal red blood cells with a concentration of  $\text{Fe}^{59}$  sufficient so that a large dilution of a small amount of the donor cells may be accurately determined by measurement of the amount of  $\text{Fe}^{59}$  present in the diluted sample.

**Conclusions.** 1. The blood volume of normal rats determined by the use of  $\text{Fe}^{59}$  labelled red blood cells is essentially the same as with  $\text{P}^{32}$  labelled cells.

2. The use of  $\text{P}^{32}$  labelled red blood cells is hence satisfactory for the determination of blood volume.

3. Determinations with  $\text{P}^{32}$  indicate that the blood volume of the normal rat is  $4.59 \pm 0.57/\text{cc}$  100 g body weight; the total red cell volume  $2.16 \pm 0.20$  cc/100 g body weight; and the hematocrit  $45.8 \pm 2.6$ .

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## 17125. The Xanthine Oxidase Activity of Rat Tissues.\*

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The scattered distribution of xanthine oxidase in various organs of different species has been summarized by Morgan.<sup>1</sup> In general, it is absent from embryonic tissue, but appears shortly after birth. It is present in the livers of most, but not all, species and is encountered frequently in kidney, spleen, lung, small intestine and occasionally in pancreas. It has not been detected in muscle, thymus, stomach or large intestine of those species studied.

The purpose of the present communication is to consider 1) the relative amounts of xanthine oxidase in various rat tissues, and 2) the changes in the enzyme activity of these tissues as the liver xanthine oxidase is depleted by dietary means.

**Methods.** Adult rats weighing 200-300 g were used throughout. Normal xanthine oxidase levels were obtained in rats maintained on Purina dog chow. Liver xanthine oxidase was depleted by feeding rats a purified 8% casein diet<sup>2</sup> for 4 to 8 weeks. The xanthine oxidase activity of various organs was determined by the method of Axelrod and Elvehjem.<sup>3</sup> Added xanthine oxidase was prepared from milk by the method of Ball.<sup>4</sup>

**Results.** Table I shows the average xanthine

oxidase activity of the normal rat tissues studied, together with the effect of adding methylene blue or purified xanthine oxidase. The amount of enzyme present in each tissue giving rise to the activity actually found could not be evaluated by a direct comparison of activities. Added xanthine oxidase was recovered nearly quantitatively when added to lung, spleen, or kidney, but was recovered in increased amounts when added to liver; hence a given weight of enzyme was more active in liver than in the other tissues. The enzyme was destroyed in the presence of homogenized small intestine; not only was the added xanthine oxidase not recovered, but in many determinations the activity fell off rapidly. All values for small intestine were therefore minimal, but alterations with diet were comparable because they were all determined in the same way.

The effect of adding 0.15 cc of 0.0113 M methylene blue in the aerobic determination of xanthine oxidase was not the same for all tissues. When added to the isolated milk xanthine oxidase or to liver homogenate, methylene blue increased the activity by an average of 3.0 and 1.6 times respectively.

TABLE I.  
Xanthine Oxidase Activity of Normal Rat Tissues.

	No. of deter- minations	Xanthine oxidase activity						
		CmmO <sub>2</sub> /g dry wt/hr			CmmO <sub>2</sub> /20 min.			
		Range	Mean ± S.E.	M. blue added	Present in tissue	X.O. added	Total found	X.O. recovered
Lung	7	395-630	479 ± 30	500	12	17	27	15
Spleen	8	390-665	534 ± 35	525	13.5	17	29	15.5
Kidney	7	0-195	135 ± 23	345	3.5	17	18.5	15
Small intestine	8	450-810	628 ± 42	1150	14	17	17	3
Stomach	6	0-85	42 ± 19	120	—	—	—	—
Liver	12	1475-2320	1862 ± 75	2790	53	18.5	83	30

\* Aided by a grant from the Nutrition Foundation, Inc.

<sup>1</sup> Morgan, E. J., *Biochem. J.*, 1926, **20**, 1282.

<sup>2</sup> Westerfeld, W. W., and Richert, Dan A.,

*Science*, 1949, **109**, 68.

<sup>3</sup> Axelrod, A. E., and Elvehjem, C. A., *J. Biol. Chem.*, 1941, **140**, 725.

<sup>4</sup> Ball, E. G., *J. Biol. Chem.*, 1939, **128**, 51.

## SERUM CHOLINESTERASE VALUES IN THYROID DISEASE

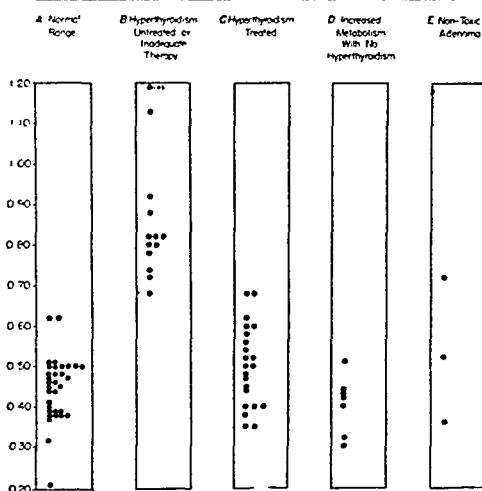


FIG. 2.

quent cholinesterase levels during adequate therapy. There appeared to be no correlation between the severity of the hyperthyroidism and the level of serum cholinesterase.

In 7 additional cases determinations were made only after adequate therapy had been instituted, so that a total of 14 cases was observed after good clinical response to antithyroid treatment. The values of this group of 14 cases were uniformly below 0.70 unit and varied from 0.35 to 0.68 unit. There were 3 types of cases in this category, 1) iodine and thyroidectomy (3 cases), 2) 6N-propyl-thiouracil, iodine and thyroidectomy (6 cases), 3) 6N-propyl-thiouracil alone (5 cases). All cases regardless of the type of therapy reacted in the same manner. In Fig. 2 is presented a summary of the results obtained in all cases.

Three cases of non-toxic adenoma were observed. In 2, normal values were obtained; a definitely high value was found in the third case. This finding is at present unexplained.

Of interest were the following 3 cases with increased basal metabolic rate in whom a clinical diagnosis of hyperthyroidism was initially entertained but subsequently ruled out.

1. Female, age 55; severe psychoneurosis, BMR plus 67%

2. Female, age 67: metastatic carcinoma of cervical lymphnodes with tracheal pressure symptoms; BMR plus 63, 66%.

3. Female, age 49; dyspnea, palpitation, fibrillation and pedal edema; BMR plus 52%, plus 63%; no response to 6N-propyl-thiouracil. Normal values for serum cholinesterase were found in each case.

No relationship was found between the serum cholinesterase level and BMR, cephalin cholesterol flocculation test, total serum protein or albumin and globulin. In this respect, our findings conflict with those of Faber,<sup>7</sup> who found a direct relationship between the cholinesterase activity and serum albumin. A tendency to an inverse relationship existed between the levels of the enzyme and serum total cholesterol.

**Discussion.** The data presented demonstrates an increase in the cholinesterase of the serum of hyperthyroid patients. Its significance is at present uncertain. In those cases in which 6N-propyl-thiouracil was used preoperatively, the serum cholinesterase returned to normal values concomitant with the improvement in clinical state and subsequent thyroidectomy did not cause a further decrease.

**Summary.** Observations on 18 cases of hyperthyroidism are presented. This condition is characterized by significantly increased levels of serum cholinesterase. These values returned toward the normal range with adequate antithyroid therapy. Of 3 patients with non-toxic adenoma of the thyroid, 2 were in the normal range and one showed a high value. No definite relationship was found between serum cholinesterase and basal metabolic rate, total serum protein, albumin, globulin or cephalin-cholesterol flocculation test. Due to the comparatively small series no conclusion can be drawn concerning sex differences in serum cholinesterase.

<sup>7</sup> Faber, M., *Acta Med. Scand.*, 1943, **114**, 72.

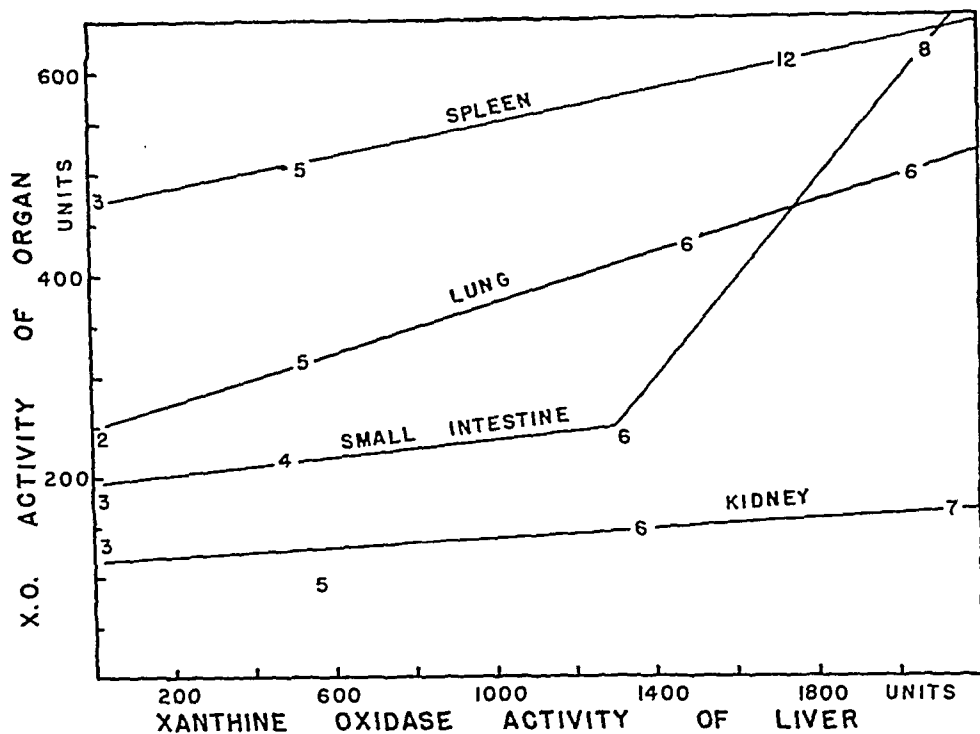


FIG. 2.

Changes in the xanthine oxidase activity of spleen, lung, small intestine and kidney as compared with the changes produced in liver xanthine oxidase by feeding a purified 8% casein diet. Numbers along curves refer to the number of determinations that were averaged to obtain each point on the curve.

of these various tissues in comparison with the changes observed in the liver.

Individual rats varied greatly in length of time on the diet required to deplete the liver of xanthine oxidase. At 4 to 5 weeks, 11 rats had 0, 0, 0, 0, 385, 395, 455, 635, 705, 1160, and 1580 units of liver xanthine oxidase; after 8 weeks, 4 of 5 rats had no liver xanthine oxidase activity. Inanition, which is known to decrease the liver xanthine oxidase,<sup>5</sup> was not a factor in these experiments with adult rats, since body weights increased consistently on the purified low protein diet. Rats were analyzed arbitrarily after 2 to 8 weeks on the diet, and the results were then correlated on the basis of the liver xanthine oxidase found; the data from rats with similar liver xanthine oxidase activities were grouped together without regard to the duration of the individual dietary periods.

As the liver xanthine oxidase activity fell from over 2000 units to zero the small intestine lost about 2/3 of its activity rapidly, but then remained fairly constant at the lower level; lung lost about 1/2 of its activity in a gradual manner; losses in the spleen and kidney were 20-30%, which were not enough to be statistically different from the normal levels in this limited series. That portion of the enzyme which could be lost readily from the small intestine was depleted more rapidly than the liver. The xanthine oxidase activities of the other tissues were much more refractory to dietary changes than was the liver enzyme.

Assuming that the intact structures maintain the same relative enzyme activities as exhibited in the homogenates, then the total xanthine oxidase activity of a normal mature rat would be distributed approximately as follows: 67% in liver, 20% in skin, 8%

<sup>5</sup> Miller, L. L., *J. Biol. Chem.*, 1948, **172**, 113.

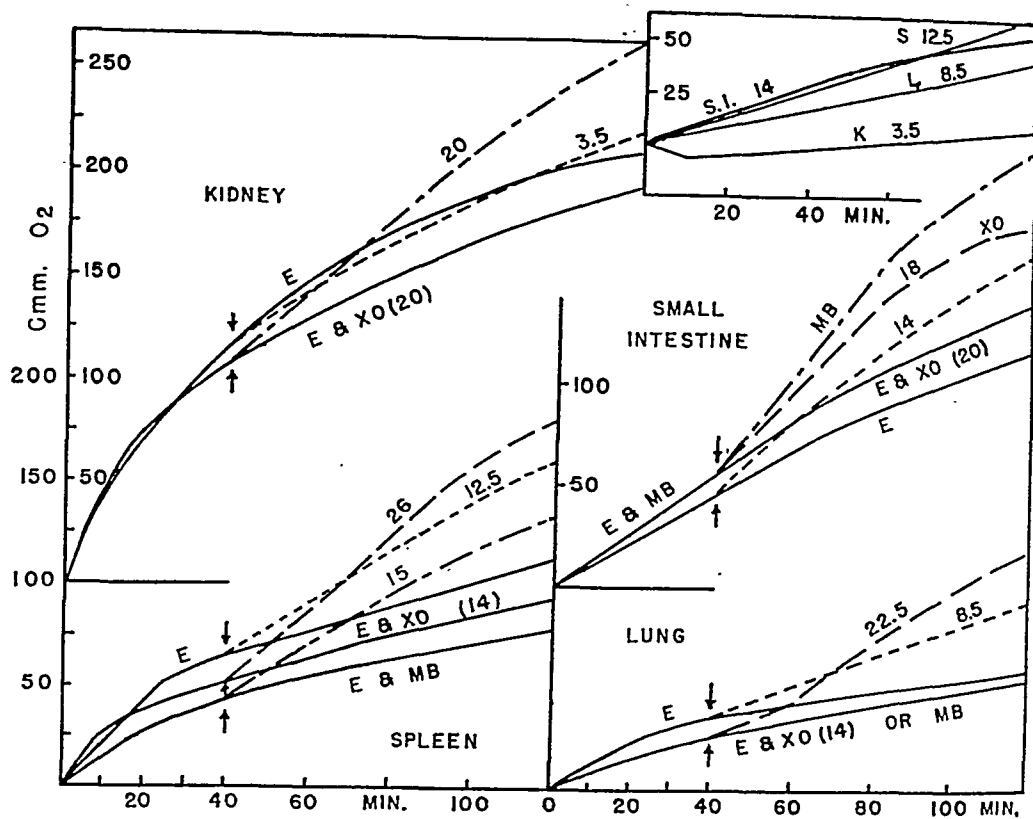


FIG. 1.

Typical oxygen consumption curves for kidney, spleen, small intestine and lung during the determination of xanthine oxidase activity. The effect of adding purified xanthine oxidase or methylene blue to the main body of the Warburg flask is also illustrated.

Solid lines = Endogenous respiration (E) with or without added xanthine oxidase (X.O.) or methylene blue (MB).

Dotted lines = Respiration after tipping in xanthine at the arrows.

Numbers along curves refer to net oxygen uptake in cmm per 20 minutes due to oxidation of xanthine.

Box, upper right: Net oxygen consumption curves due to oxidation of xanthine.

Kidney, small intestine and stomach also gave appreciable increases in xanthine oxidase activity in the presence of methylene blue, and this was very useful in demonstrating the presence of this enzyme in kidney and stomach where the amount was often so small as to be questionable. The xanthine oxidase activity of lung and spleen were unchanged in the presence of methylene blue. The significance of the differences between tissues in respect to this methylene blue effect is not yet apparent.

Brain, skeletal muscle and testes were devoid of xanthine oxidase activity by the usual test as well as in the presence of methylene blue aerobically. Skin contained 100 to 200

units of xanthine oxidase activity (CmmO<sub>2</sub>/g/hr), and methylene blue increased the activity. Skin xanthine oxidase was not studied in detail because of the difficulty in homogenizing this tissue.

Fig. 1 shows typical oxygen consumption curves for kidney, spleen, lung and small intestine during the determination of xanthine oxidase activity. The effect of adding purified xanthine oxidase or methylene blue to the main body of the Warburg flask is also illustrated. Similar studies with liver will be reported in another communication.

Fig. 2 shows the effect of a purified low-protein diet on the xanthine oxidase activity

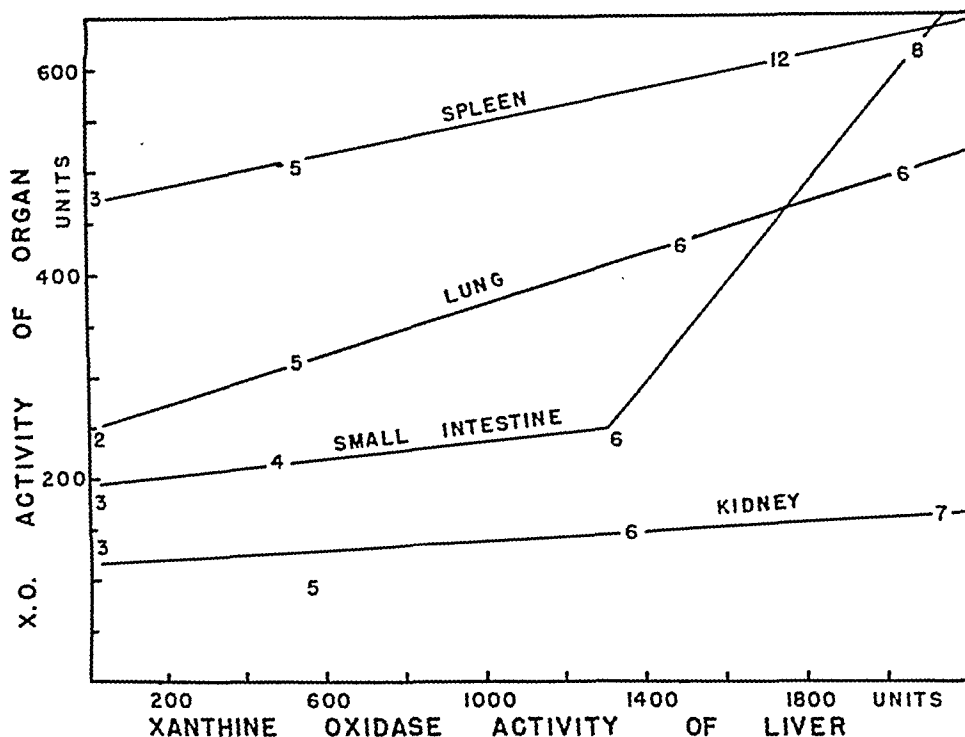


FIG. 2.

Changes in the xanthine oxidase activity of spleen, lung, small intestine and kidney as compared with the changes produced in liver xanthine oxidase by feeding a purified 8% casein diet.

Numbers along curves refer to the number of determinations that were averaged to obtain each point on the curve.

of these various tissues in comparison with the changes observed in the liver.

Individual rats varied greatly in length of time on the diet required to deplete the liver of xanthine oxidase. At 4 to 5 weeks, 11 rats had 0, 0, 0, 0, 385, 395, 455, 635, 705, 1160, and 1580 units of liver xanthine oxidase; after 8 weeks, 4 of 5 rats had no liver xanthine oxidase activity. Inanition, which is known to decrease the liver xanthine oxidase,<sup>5</sup> was not a factor in these experiments with adult rats, since body weights increased consistently on the purified low protein diet. Rats were analyzed arbitrarily after 2 to 8 weeks on the diet, and the results were then correlated on the basis of the liver xanthine oxidase found; the data from rats with similar liver xanthine oxidase activities were grouped together without regard to the duration of the individual dietary periods.

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Assuming that the intact structures maintain the same relative enzyme activities as exhibited in the homogenates, then the total xanthine oxidase activity of a normal mature rat would be distributed approximately as follows: 67% in liver, 20% in skin, 8%

<sup>5</sup> Miller, L. L., *J. Biol. Chem.*, 1948, **172**, 113.

in small intestine, 2% each in spleen and lung, and 1% in kidney. When the liver has been depleted to zero xanthine oxidase activity by a purified low protein diet, the entire rat has lost about 75% of its original enzyme activity (assuming the skin is relatively unchanged).

**Summary.** The average xanthine oxidase activities in  $\text{CmmO}_2/\text{g}$  dry weight/hour for adult rat tissues were: Liver 1862, small intestine 628, spleen 534, lung 479, kidney 135, stomach 42, skin 100-200, brain 0, muscle 0, testes 0. Two-thirds of the total activity in the entire rat was in the liver.

Methylene blue added to the Warburg flask

in the aerobic determination increased the xanthine oxidase activity in liver, kidney, stomach and small intestine, but not in lung or spleen. Added xanthine oxidase was recovered quantitatively in the presence of lung, spleen and kidney; greater than added amounts of activity were recovered from liver and decreased amounts from small intestine.

As the liver xanthine oxidase was depleted by a purified low protein diet, the small intestine lost about  $2/3$  of its activity, and lung lost about  $1/2$ . Losses from the spleen and kidney were small. The entire rat lost  $3/4$  or more of its xanthine oxidase activity.

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## 17126. Relationship Between Protein Osmotic Pressure and Density in Plasma from Cats, Dogs, and Humans.

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During the course of experiments on the effective osmotic pressure of the plasma proteins in the capillary circulation<sup>1</sup> we have had occasion to compare protein osmotic pressure with density in a large number of samples of plasma from cats, dogs, and humans. We were surprised to find that for any given density the protein osmotic pressure of human plasma is higher than that of plasma from cats or dogs. The data given below may prove useful to other investigators who wish to use plasma density as a measure of the osmotic activity of the plasma proteins in these species.

**Methods.** Blood was drawn from the carotid arteries of cats and dogs anesthetized with Nembutal. Human blood was obtained by venipuncture. Heparin was used to prevent coagulation. Low protein concentrations were obtained by diluting the plasma with

Ringer's solution (0.9% NaCl, 0.042% KCl, 0.024%  $\text{CaCl}_2$ , 0.020%  $\text{NaHCO}_3$ ) of density 1.0068 relative to water at the same temperature. High protein concentrations were obtained by evaporating the plasma in cellophane bags placed in front of a fan. The bags were then sealed close to the liquid level and the concentrated plasma dialyzed against cold Ringer's solution until ionic equilibrium was established as indicated by the electrical conductivity.

Densities were determined by the Falling Drop Method of Barbour and Hamilton.<sup>2</sup> The standard deviation from the means of 68 duplicate determinations performed with this method was  $\pm 0.00013$  g/cc. Protein osmotic pressure measurements were made across collodion membranes using a Hepp osmometer.<sup>3</sup> All the results were corrected to  $37^\circ\text{C}$  by multiplying the observed values by the factor  $273 + 37 \div 373 + t$ , in which  $t = \text{room}$

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<sup>1</sup> Pappenheimer, J. R., and Soto-Rivera, A., *Am. J. Physiol.*, 1948, 152, 471.

<sup>2</sup> Barbour, H. G., and Hamilton, W. F., *J. Biol. Chem.*, 1926, 69, 625.

<sup>3</sup> Hepp, O., *Z. f. d. Gesamte Exp. Med.*, 1936, 99, 709.

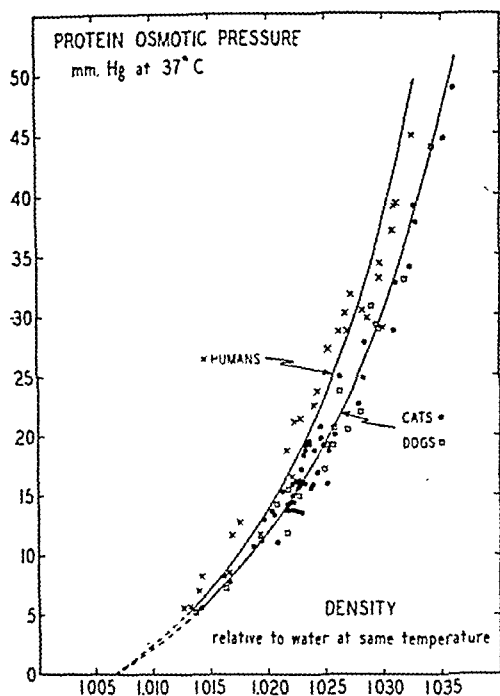


FIG. 1.

temperature °C. The standard deviation from the means of 68 duplicate measurements by this method was  $\pm 0.4$  mm Hg. The pH of the plasma samples ranged from 7.2 - 7.5 (glass electrode). For purposes of calculation, however, a value of 7.4 was assumed since a variation of  $\pm 0.3$  pH unit produces a change of protein osmotic pressure too small to be detected with our apparatus (cf. also Equation 1)

**Results.** Fig. 1 shows the relations between density and protein osmotic pressure in the three species. It is seen that at any given density human plasma has a higher protein osmotic pressure than that of cat or dog plasma. There is no significant difference in this respect between cat plasma and dog plasma.

**Discussion.** The following analysis of the data leads to useful formulae relating protein osmotic pressure to density and provides a basis for interpretation of the results.

As shown by Scatchard *et al.*<sup>4,5</sup> protein

osmotic pressure is related to protein concentration by an equation of the form:

$$\pi = \frac{\pi_0 (1 - .649) C}{1 - (.004 + .009pH)C} \quad (1)$$

where  $\pi$  = protein osmotic pressure mm Hg,  $\pi_0$  = protein osmotic pressure per gram protein at infinite dilution,  $g$  = ratio of globulin to total protein and  $C$  = total protein concentration in g/100 cc. For human plasma at pH 7.4 the constants are such that

$$\pi = \frac{1.97C}{1 - 0.071C} \quad (2)$$

in which  $C$  is assumed to be 6.25 times the protein nitrogen. Protein concentration is related to density by an equation of the form:

$$C = K (\varphi - \varphi_0) \quad (3)$$

in which  $\varphi_0$  is the density at zero protein concentration. In the present experiments in which plasma was diluted with Ringer's solution  $\varphi_0 = 1.0068$

Substituting Equation 3 into Equation 2 we have

$$\pi = \frac{1.97 K (\varphi - 1.0068)}{1 - 0.071K (\varphi - 1.0068)} \quad (4)$$

The curve conforming to Equation 4 which best fits our experimental data for human plasma (method of least squares) is drawn in Fig. 1. It leads to a value for  $K = 353$  which may be compared with values ranging from 343-360 found by previous investigators who compared protein concentration (actually protein nitrogen) with density.<sup>6-9</sup> For the practical purpose of predicting protein osmo-

<sup>6</sup> Moore, N. S., and VanSlyke, D. D., *J. Clin. Invest.*, 1930, **8**, 337.

<sup>7</sup> Phillips, R. A., VanSlyke, D. D., Dole, V. P., Emerson, K., Hamilton, P. B., and Archibald, R. M., Copper sulfate method for measuring specific gravities of whole blood and plasma, Josiah Macy, Jr. Foundation, New York, 1945.

<sup>8</sup> Lowry, O. H., and Hunter, T. H., *J. Biol. Chem.*, 1945, **159**, 465.

<sup>9</sup> Kagan, B. M., *J. Clin. Invest.*, 1938, **17**, 373.

<sup>4</sup> Scatchard, G., Batchelder, A. G., and Brown, A., *J. Clin. Invest.*, 1944, **23**, 459.

<sup>5</sup> Scatchard, G., private communication.



tic pressure from density in human plasma Equation 4 becomes

$$\pi = \frac{695 (\rho - 1.0068)}{1 - 25 (\rho - 1.0068)} \pm 2.4 \text{ mm Hg (5)}$$

An examination of the partial specific volumes of the purified protein components of plasma by Oncley, Scatchard and Brown<sup>10</sup> has shown that the average specific volume of the proteins is not less than 0.74 cc/g. Since  $K$  is related to specific volume ( $V\sigma$ ) by the relation

$$K = \frac{1}{1 - \rho_0 V\sigma} \quad \dots\dots\dots(6)$$

we may predict that the minimum value of  $K$  for whole plasma is 389 and not  $350 \pm 10$ , as found in this and previous investigations cited above. An explanation of this discrepancy may be found in the recent studies of Armstrong, Budka, Morrison and Hasson<sup>11</sup> who report that protein in plasma is associated with (nitrogen-free) lipids so that the factor relating protein concentration to protein nitrogen in human plasma is closer to 6.8 than 6.25. Correction of  $K$  by the factor  $6.8/6.25$  yields a value of  $K = 6.8/6.25 \times 353 = 385$  which is in close agreement with the value 389 predicted from the partial specific volumes of the individual components.

A similar analysis for dog and cat plasma cannot at present be made because the constants essential to Equation 2 have not been

evaluated and because the effects of lipids on the factor relating protein nitrogen to protein concentration has not been determined for these species. However, for the practical purpose of calculating the osmotic pressure from density it is reasonable to suppose that the equation is of the same form as Equation 5. The curve defined by an equation of this form which best fits our experimental data for cat and dog plasma is drawn in Fig. 1. For these species

$$\pi = \frac{625 (\rho - 1.0068)}{1 - 22.5 (\rho - 1.0068)} \pm 1.7 \text{ mm Hg } \dots\dots(7)$$

The observed differences between human plasma and cat and dog plasma shown in Fig. 1 and in Equations 5 and 7 are in the direction to be expected if cat and dog plasma contain more lipid associated with plasma protein than does human plasma. Until further data are available, however, it is not possible to state that this is the sole factor involved.

**Summary.** 1. Data are given showing the relationship between protein osmotic pressure and density in plasma from normal humans, cats, and dogs. Formulae are derived which allow the prediction of protein osmotic pressure from density in each of the three species (Equations 5 and 7). 2. For any given density human plasma has a higher protein osmotic pressure than does plasma from cats or dogs. Possible reasons for this species difference are discussed.

I wish to express my gratitude to Dr. J. R. Pappenheimer for his invaluable help and suggestions throughout this work.

Received April 11, 1949. P.S.E.B.M., 1949, 71.

<sup>10</sup> Oncley, J. L., Scatchard, G., and Browne, A., *J. Phys. and Colloid Chem.*, 1947, **51**, 184.

<sup>11</sup> Armstrong, S. H., Budka, M. J. E., Morrison, K. C., and Hasson, M., *J. Am. Chem. Soc.*, 1947, **69**, 1747.

# 17127. Xanthine and Mercurial Diuretics and Renal Tubular Transport of Glucose and P-aminohippurate in the Dog.\*

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Mercurial and probably xanthine diuretics<sup>1</sup> inhibit certain enzyme systems in the kidney. It is probable that the diuretic action of these agents is due to their selective accumulation in the kidney in concentrations sufficient to inhibit the systems concerned with the re-

absorption of water and sodium chloride. The action of these compounds on other renal transport mechanisms has not received much attention. It has been reported<sup>2,3</sup> that mersalyl (Salyrgan) depresses the maximal rate of transfer of diodrast and p-aminohippurate

TABLE I.  
Effects of Salyrgan and Mercuhydrin on Transfer Maxima of Glucose and PAH.

Dog No.	Wt (kg)	Date	Control			During diuresis		
			GF* cc/min.	TmG* mg/min.	TmPAH* mg/min.	GF* cc/min.	TmG* mg/min.	TmPAH* mg/min.
Tm-1	17	7- 2-48	53	212		62	228	
1	17	11-23-	65	245		79	254	
1	17	12-17-	54		11	61		14
1	17	1-13-49	61		10	70		13
2	17	-20-	63		14	68		15
3	15	10-22-48	65	215		63	198	
3	15	11- 1-	52		9	44		10
3	15	12- 3-	56	196		64	220	
3	15	-29-	53		10	63		10
4	15	11-18-	67	232		61	220	
6	14	7-16-	47	233		42	228	
6	14	-22-	42		10	45		9
7	16	- 9-	60	225		47	205	
7	16	8-18-	55	200		48	172	
7-F	15	4-29-	52	193		49	180	

\* Average of three 10-minute periods.

TABLE II.  
Effects of Theophylline on Transfer Maxima of Glucose and PAH.

Dog No.	Wt (kg)	Date	Control			During diuresis		
			GF* cc/min.	TmG* mg/min.	TmPAH* mg/min.	GF* cc/min.	TmG* mg/min.	TmPAH* mg/min.
Tm-1	15	7- 2-48	66		10	58		11
1	15	- 7-	53	212		60	230	
1	15	12-17-	50		11	57		14
3	15	9- 9-	53		11	57		12
3	15	-16-	51	187		47	173	
6	14	7-16-	47	232		42	225	
6	14	-23-	52		11	58		10
7	17	6-23-	71	261		64	242	
6-F	16	11-13-	60	252		62	270	

\* Average of three 10-minute periods.

\* This investigation was supported in part by a research grant from the Division of Research Grants and Fellowships of the National Institute of Health, United States Public Health Service.

† Handley, C. A., unpublished data.

<sup>2</sup> Brun, C., Hilden, T., and Rauschow, F., *Acta Pharmacol. Toxicol.*, 1947, **3**, 1.

<sup>3</sup> Berliner, R. W., Kennedy, T. J., and Hilton, J. G., *Am. J. Physiol.*, 1948, **154**, 537.

tic pressure from density in human plasma Equation 4 becomes

$$\pi = \frac{695 (\rho - 1.0068)}{1 - 25 (\rho - 1.0068)} \pm 2.4 \text{ mm Hg} \quad (5)$$

An examination of the partial specific volumes of the purified protein components of plasma by Oncley, Scatchard and Brown<sup>10</sup> has shown that the average specific volume of the proteins is not less than 0.74 cc/g. Since  $K$  is related to specific volume ( $V\sigma$ ) by the relation

$$K = \frac{1}{1 - \rho_0 V\sigma} \quad \dots\dots\dots (6)$$

we may predict that the minimum value of  $K$  for whole plasma is 389 and not  $350 \pm 10$ , as found in this and previous investigations cited above. An explanation of this discrepancy may be found in the recent studies of Armstrong, Budka, Morrison and Hasson<sup>11</sup> who report that protein in plasma is associated with (nitrogen-free) lipids so that the factor relating protein concentration to protein nitrogen in human plasma is closer to 6.8 than 6.25. Correction of  $K$  by the factor 6.8/6.25 yields a value of  $K = 6.8/6.25 \times 353 = 385$  which is in close agreement with the value 389 predicted from the partial specific volumes of the individual components.

A similar analysis for dog and cat plasma cannot at present be made because the constants essential to Equation 2 have not been

evaluated and because the effects of lipids on the factor relating protein nitrogen to protein concentration has not been determined for these species. However, for the practical purpose of calculating the osmotic pressure from density it is reasonable to suppose that the equation is of the same form as Equation 5. The curve defined by an equation of this form which best fits our experimental data for cat and dog plasma is drawn in Fig. 1. For these species

$$\pi = \frac{625 (\rho - 1.0068)}{1 - 22.5 (\rho - 1.0068)} \pm 1.7 \text{ mm Hg} \quad \dots\dots\dots (7)$$

The observed differences between human plasma and cat and dog plasma shown in Fig. 1 and in Equations 5 and 7 are in the direction to be expected if cat and dog plasma contain more lipid associated with plasma protein than does human plasma. Until further data are available, however, it is not possible to state that this is the sole factor involved.

**Summary.** 1. Data are given showing the relationship between protein osmotic pressure and density in plasma from normal humans, cats, and dogs. Formulae are derived which allow the prediction of protein osmotic pressure from density in each of the three species (Equations 5 and 7). 2. For any given density human plasma has a higher protein osmotic pressure than does plasma from cats or dogs. Possible reasons for this species difference are discussed.

I wish to express my gratitude to Dr. J. R. Pappenheimer for his invaluable help and suggestions throughout this work.

Received April 11, 1949. P.S.E.B.M., 1949, 71.

<sup>10</sup> Oncley, J. L., Scatchard, G., and Browne, A., *J. Phys. and Colloid Chem.*, 1947, **51**, 184.

<sup>11</sup> Armstrong, S. H., Budka, M. J. E., Morrison, K. C., and Hasson, M., *J. Am. Chem. Soc.*, 1947, **69**, 1747.

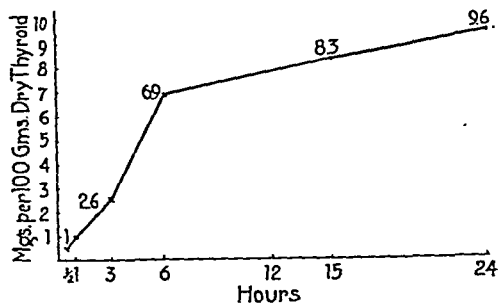


FIG. 1.

Manganese deposition in the thyroid gland of the guinea pig following a single subcutaneous injection of manganese chloride in a dosage of 10.0 mg per kg of body weight.

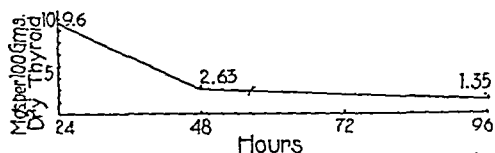


FIG. 2.

Rate of elimination of manganese from the thyroid gland of the guinea pig.

content of the thyroid gland was determined by the procedure of Ray.<sup>2</sup> The results are recorded graphically in Fig. 1 and 2. Each point on the graphs represents the average amount of manganese found in the thyroids of 3 animals. A detectable increase over the

level found in earlier tests<sup>1</sup> was noted in 30 minutes, and a measurable increment was found in one hour. Fig. 1 shows further that the accumulation of manganese was rapid during the early stages of absorption. Of the total amount deposited in a 24 hour period, about 70% of it accumulated within the first 6 hours. Fig. 2 shows that the elimination of manganese from the thyroid, once it began, was very rapid. More than 70% of the 24 hour accumulation disappeared within 48 hours after the injection, and 85% of it disappeared within 96 hours.

**Summary.** Subcutaneous injection of manganese chloride into the guinea pig caused manganese to accumulate in all organs of the body tested, but the deposition was especially great in the thyroid gland where it began to appear within 30 minutes after administration. Absorption was particularly rapid during the first few hours, but the element continued to accumulate for at least 24 hours. Of the total amount of manganese deposited in the thyroid gland about 70% accumulated during the first 6 hours. Elimination of manganese from the thyroid, once it began, was rapid. Seventy per cent of the 24 hour accumulation disappeared within 48 hours after the injection; 85% of it vanished in 96 hours.

Received April 12, 1949. P.S.E.B.M., 1949, 71.

<sup>2</sup> Ray, T. W., *J. Biol. Chem.*, 1940, 134, 677.

## 17129. Effect of the D- and L-isomers of Isoamidone\* on the Permeability of Dog Erythrocytes.†

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We have previously reported that changes in the permeability of erythrocytes *in vitro* appeared to be correlated with changes in the activity of the acetylcholine-cholinesterase system in the red cell.<sup>1,2</sup> This conclusion was

based on the observation that physostigmine as well as methadon,<sup>§</sup> both of which are chol-

<sup>1</sup> Greig, M. E., and Holland, W. C., submitted for publication to *Arch. Biochem.*

<sup>2</sup> Greig, M. E., and Holland, W. C., *Fed. Proc.*, 1949, 8, 297.

<sup>§</sup> Methadon, amidone, 2-dimethylamino-4,4-diphenyl-heptanone 5. The methadon and isoamidone were kindly supplied by the Mallinckrodt Chemical Works.

\* 1-dimethylamino-2-methyl-3,3-diphenyl hexanone-4.

† Funds for carrying out this work were kindly supplied by the Mallinckrodt Chemical Works.

‡ U. S. Public Health Fellow.

(PAH) in man although the TmPAH in the dog is not influenced by mersalyl.<sup>3</sup> The following data concerns the measurement of glucose and PAH Tm's in normal dogs and during the period of maximal diuresis by mercurial diuretics and theophylline.

**Methods.** Trained, unanesthetized, female dogs weighing 14-17 kg were used. Glomerular filtration rate (GF) was measured by creatinine. Glucose and p-aminohippurate Tm's were measured in independent experiments because of the reported mutual interference in the tubular transfer of these substances.<sup>4,5</sup>

Creatinine was determined by the method of Folin and Wu,<sup>6</sup> p-aminohippurate by the method of Bratton and Marshall<sup>7</sup> and glucose by Hanes' modification of Hagedorn and Jensen's method.<sup>8</sup> The dose of meralluride (Mer-

cuhydrin) and mersalyl, the two mercurials used, was 0.1 cc per kg (4 mg per kg of combined mercury) and 10-20 mg per kg of theophylline was employed.

**Results.** The results recorded in Tables I and II show that neither theophylline nor the mercurial diuretics studied interfere with the renal tubular transfer mechanisms for glucose or p-aminohippurate. These results indicate that the energy yielding mechanism concerned with the reabsorption of water and sodium chloride in the dog is distinct from the mechanisms concerned in the transfer of glucose and PAH. During this investigation it was observed that it is possible to reduce the TmG and TmPAH indirectly by diuretics.<sup>1</sup> This effect is due to dehydration and may be produced by the saline diuretics as well as the mercurial and xanthine groups, if sufficient urine output is produced to cause dehydration.

**Summary.** Mercuhhydrin, salyrgan and theophylline, in diuretic doses, do not interfere with the renal tubular transfer of glucose or p-aminohippurate in the dog.

Received April 22, 1949. P.S.E.B.M., 1949, 71.

<sup>4</sup> Klopp, C., Young, N. F., and Taylor, H. D., *J. Clin. Invest.*, 1945, **24**, 117.

<sup>5</sup> Houek, C. R., *Proc. Soc. Exp. Biol. and Med.*, 1946, **63**, 398.

<sup>6</sup> Folin, O., and Wu, H. J., *J. Biol. Chem.*, 1919, **38**, 81.

<sup>7</sup> Bratton, A. C., and Marshall, E. K., *J. Biol. Chem.*, 1939, **128**, 537.

<sup>8</sup> Hanes, C. S., *Biochem. J.*, 1929, **23**, 99.

## 17128. Absorption of Manganese by the Thyroid Gland of the Guinea Pig.

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Ray and Deysach<sup>1</sup> found that subcutaneous injection of manganese chloride into guinea pigs resulted in the accumulation of manganese in varying amounts in all organs tested, but that the accumulation was especially great in the thyroid gland where deposition was in direct proportion to the dose administered. Injections of large doses of the salt depressed the oxygen consumption of the animal and small doses augmented it. Noticeable effects within a few minutes after administration suggested early absorption by the thy-

roid gland. The purposes of the present study were to find the exact time interval for the appearance of manganese in the thyroid following its subcutaneous injection, to determine the rate at which it is deposited in this organ during a 24 hour period, and finally to obtain information on its rate of elimination.

**Experimental.** Each of 27 guinea pigs was given one subcutaneous injection of 10.0 mg of manganese chloride per kg of body weight. The animals were sacrificed 3 at a time, at intervals ranging from 15 minutes to 4 days following the injection, and the manganese

<sup>1</sup> Ray, T. W., and Deysach, L. J., *Proc. Soc. Exp. Biol. and Med.*, 1942, **51**, 228.

In our experiments the hemoglobin liberated has been taken as a criterion for changes in permeability of erythrocytes. If percent hemolysis be plotted against time, six possible arrangements of curves representing the effects of the D- and L-isomers of isoamidone on erythrocytes could be obtained. If C represents the hemolysis in the control suspension, D- that with the D-isomer and L- that with the L-isomer the 6 possible types obtainable would be (1)  $L > D > C$ , (2)  $D > L > C$ , (3)  $C > D > L$ , (4)  $C > L > D$ , (5)  $D > C > L$ , (6)  $L > C > D$ .

Experimentally we found that all 6 types occurred to some extent under different conditions. The factors controlling the type of curve obtained appeared to be mainly the proportions of Na and K in the medium and the pH. There seemed to be some variation in results obtained with red cells from different dogs under the same experimental conditions, although the results obtained with different samples of blood from the same dog under the same experimental conditions were quite consistent.

In a medium containing a high proportion of KCl compared with NaCl, curves of type 3 were most frequently obtained, while those of type 5 and type 1 occurred less often. Of 23 experiments carried out in a medium consisting of isotonic KCl or a mixture of isotonic KCl and isotonic NaCl in the proportions of 9:1, fifteen fell in type 3 and 8 in type 5. It was frequently observed that early in an experiment the results would follow curves of type 3, later the curves would cross and follow those of type 5 and still later those of type 1 and occasionally type 2.

In a medium containing a high proportion of Na compared with K there was little difference in the effects produced by D- and L-isomers. Usually curves of types 1 and 2 were obtained.

Results of 3 typical experiments are presented graphically in Fig. 1-3.

**Discussion.** It may be seen from the above results that:

1. the D- and L-isomers of isoamidone in the same concentrations inhibit the glycolysis of glucose by rat brain and by dog erythrocytes to about the same extent.

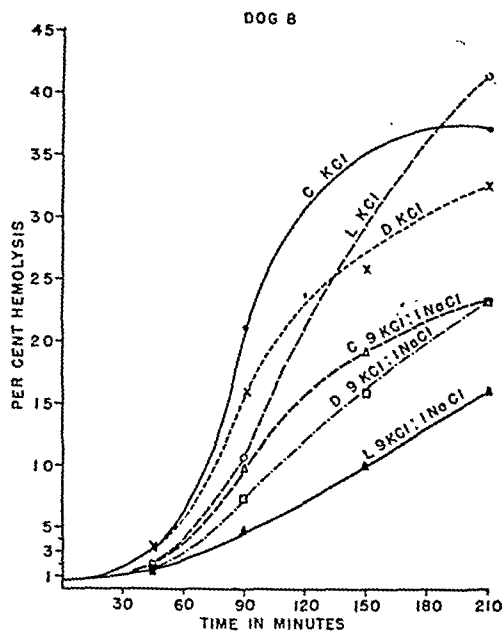


FIG. 1.

One cc packed dog erythrocytes were suspended in 10 cc isotonic saline in Erlenmeyer flasks. The final concentration of the isoamidone was 0.00022 M, that of acetylcholine 0.01 M. Samples of suspension of 1 cc were removed at intervals and after dilution and centrifugation the hemoglobin content of the supernatant fluid was determined using a photoelectric colorimeter.

C—Control.

D—D-isoamidone.

L—L-isoamidone.

Ac ch—Acetyl choline.

2. the D- and L-isomers of isoamidone inhibit the cholinesterase activity of erythrocytes to different extents, the L- being a more effective inhibitor than the D-isomer.

3. the D- and L-isomers of isoamidone have different effects on the permeability of dog erythrocytes.

As the stereoisomers have the same chemical properties, *e.g.*, molecular weight, solubility, molecular radius, rate of diffusion, the different effects on the permeability of erythrocytes could not be attributed to these factors.

If one assumes that the permeability of living cells is controlled by metabolic activity, then of the two metabolic reactions investigated which are affected by isoamidone the inhibition of cholinesterase activity would seem best able to account for the changes in permeability in view of the different effects pro-

TABLE I.

Effect of the D- and of the L-isomers of Isoamidone on Cholinesterase Activity of Dog Erythrocytes.

The media used were Ringer Krebs bicarbonate saline (R.K.),<sup>7</sup> sodium bicarbonate saline (Na) consisting of 100 parts 0.9% NaCl and 21 parts 1.3% NaHCO<sub>3</sub>, and potassium bicarbonate saline (K) consisting of KCl and KHCO<sub>3</sub> in the same proportions as for the sodium buffer. 0.2 cc packed dog erythrocytes, and acetylcholine in a final concentration of 0.01 M in a volume of 2 cc were used. Carbon dioxide evolution was measured in Warburg manometers.

Medium	Duration of exp., min.	Conc. of drug, M		Mm <sup>3</sup> CO <sub>2</sub> evolved				
		D	L	Control	D	% effect	L	% effect
Na	60	.006	.0002	115	84	27	84	26
K	60	.006	.0002	124	87	30	67	46
RK	60	.00087	.00087	202	124	39	98	51
RK	60	.00087	.00087	228	168	26	102	55

TABLE II.

Effect of the D- and the L-isomers of Isoamidone on Glycolysis.

The experiments were carried out in a Ringer Krebs bicarbonate medium<sup>7</sup> in Warburg manometers. The concentration of glucose in the experiment with erythrocytes was 0.014 M, that in the experiment with brain 0.01 M. The amount of tissue was 1.0 cc packed dog erythrocytes or 150 mg homogenized rat brain in a final volume of 2 cc. The gas phase was 95% N<sub>2</sub>, 5% CO<sub>2</sub>.

Tissue	Duration of exp., min.	Conc. of isoamidone, M	CO <sub>2</sub> evolved in mm <sup>3</sup>				
			Control	D	% effect	L	% effect
Erythrocytes	40	.00174	89	70	21	77	14
Rat Brain	60	.00174	130	107	18	101	22

inesterase inhibitors<sup>3,4</sup> produced changes in permeability of dog erythrocytes under certain conditions. Physostigmine is considered to be a specific inhibitor of cholinesterase. This drug produced most marked changes in the permeability of erythrocytes under the conditions where it had its maximum effect on cholinesterase activity. Methadon is not a specific inhibitor of cholinesterase as it also inhibits glycolysis of glucose<sup>5</sup> as well as certain oxidative processes.<sup>6</sup> However, under our experimental conditions, the inhibition of cholinesterase rather than the inhibition of glycolysis by methadon seemed to account for its effect on permeability of erythrocytes.

As the D- and L-isomers of isoamidone have been found to be equally effective in inhibiting

glycolysis, but the L-isomer is a much more effective inhibitor of cholinesterase than is the D, it was assumed that if the effects on permeability were different with the different isomers, this action could be more readily attributed to their effect on cholinesterase than to that on glycolysis.

The present communication describes experiments dealing with the effects of the D- and of the L-isoamidone on the permeability of dog erythrocytes.

**Results.** In Table I are presented results of experiments on the effects of the D- and L-isomers of isoamidone on the cholinesterase of dog erythrocytes. As in the case of brain cholinesterase the L-isomer has a much greater inhibitory action than has the D- in the same concentrations.<sup>3</sup>

From Table II it may be seen that there is little or no difference between the effects of the D- and L-isomers on glycolysis of glucose by rat brain or by dog erythrocytes.

<sup>3</sup> Greig, M. E., and Howell, R. S., *Proc. Soc. Exp. Biol. and Med.*, 1948, **68**, 352.

<sup>4</sup> Eadie, G. S., Bernheim, F., and Fitzgerald, D. P., *J. Pharm. Exp. Therap.*, 1948, **94**, 19.

<sup>5</sup> Greig, M. E., *Arch. Biochem.*, 1948, **17**, 129.

<sup>6</sup> Greig, M. E., and Howell, R. S., *Arch. Biochem.*, 1948, **19**, 441.

<sup>7</sup> Krebs, H. A., and Henseleit, K., *Z. physiol. Chem.*, 1932, **210**, 33.

# 17130. Interrelationship of Vitamin B<sub>12</sub> and Choline.\* I. Effect on Hemorrhagic Kidney Syndrome in the Rat.

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Following the isolation of vitamin B<sub>12</sub> by Rickes and co-workers,<sup>1</sup> several investigators<sup>2-4</sup> have reported data indicating the identity or close relation of this vitamin with the "animal protein factor." Studies by Bird, Rubin, and Groschke<sup>5</sup> have indicated that methionine can function as a partial substitute for the "animal protein factor" in soybean protein diets for chicks. In view of the relationship between methionine and choline, it seemed of interest to determine whether vitamin B<sub>12</sub> might exert a sparing action on the choline requirement of the rat.

It is the purpose of this paper to report that the incidence and the severity of renal hemorrhage in weanling rats receiving a diet low in choline and methionine were significantly decreased by supplementing the diet

with vitamin B<sub>12</sub> in concentrate or crystalline form.

*Experimental.* Weanling rats of the AES (Alabama Experiment Station) strain weighing 40-50 g were placed in individual cages and uniformly grouped with respect to number, weight, sex and litter. Feed and water were supplied *ad libitum*. The basal diet contained extracted peanut meal 30,<sup>6</sup> sucrose 39.5, extracted casein 6,<sup>7</sup> salt mixture<sup>7</sup> (undried) 4.4, L-cystine 0.1, cod liver oil 1, lard 19. Vitamins were added, mg/kg of diet, as follows: thiamine 2, pyridoxine<sup>2</sup>, riboflavin 4, calcium pantothenate 10, niacin 20, i-inositol 200, alpha-tocopherol 25, alpha-tocopherol acetate 25, and menadione 5. The total methionine and choline content of the diet was .3% and .007% respectively.

On this basal diet without added choline or methionine, a 100% incidence of fatal kidney hemorrhage occurs in weanling rats of the AES strain in 2 weeks or less. In the present experiments, various sub-protective levels of choline or methionine were added to the basal diet. The effect of adding vitamin B<sub>12</sub> in concentrate or crystalline form to the diet for each of the treatments was then determined. In one experiment the effect of adding vitamin B<sub>12</sub> concentrate to the basal diet supplemented with an adequate protective level of choline (.2%) was determined. Except in the latter experiment, all rats were necropsied after death or at the end of a 14-day experimental period, and the kidneys were carefully examined for gross pathological lesions.

The results on the effects of vitamin B<sub>12</sub> with sub-protective levels of choline are shown in Table I. At levels of .04% and .06% of choline chloride without vitamin B<sub>12</sub>, the

\* Published with the approval of the Director of the Alabama Agricultural Experiment Station. This work was supported in part by a grant from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council. It was also aided by donations of concentrates and crystalline vitamin B<sub>12</sub> and other vitamins by Merck and Co., and of folic acid by Lederle Laboratories. These data were presented in part at the Birmingham meeting of the Southern Section, Society for Experimental Biology and Medicine, February 15, 1949, and at the Detroit meeting, Fed. Am. Soc. Exp. Biol., April 20, 1949.

<sup>1</sup> Rickes, E. L., Brink, N. G., Konviszy, F. R., Wood, T. R., and Folkers, K., *Science*, 1948, **107**, 396.

<sup>2</sup> Ott, W. H., Rickes, E. L., and Wood, T. R., *J. Biol. Chem.*, 1948, **174**, 1047.

<sup>3</sup> Lillie, R. J., Denton, C. A., and Bird, H. R., *J. Biol. Chem.*, 1948, **176**, 1477.

<sup>4</sup> Nichol, C. A., Dietrich, L. G., Cravens, W. W., and Elvehjem, C. A., *Proc. Soc. Exp. Biol. and Med.*, 1949, **70**, 40.

<sup>5</sup> Bird, H. R., Rubin, M., and Groschke, A. C., *J. Nutrition*, 1947, **33**, 319.

<sup>6</sup> Engel, R. W., *J. Nutrition*, 1948, **36**, 739.

<sup>7</sup> Salmon, W. D., *J. Nutrition*, 1947, **33**, 155.



duced by the two isomers on this enzyme.

In a medium containing a high proportion of KCl compared with NaCl the L-isomer usually produced greater resistance to hemolysis than the D at the start of the experiment, (type 3 and Fig. 1).

In a bicarbonate medium, pH 8, the L usually increased hemolysis, relative to the control and the D-isomer (Fig. 2). These results are similar to those found with physostigmine.<sup>1,2</sup> Occasionally curves of type 5 were found early in the experimental period, in which the L-isomer caused increased resistance and the D-increased fragility relative to the control.

The addition of acetylcholine increases the resistance of the control suspension in a K medium and tended to increase the differences between the effects produced by the D- and L-isomers relative to the control (Fig. 2).

In a medium consisting of either NaCl or high Na compared with K the differences between the effects of the D- and L-isomers were usually less marked (Fig. 3) and the D occasionally produced slightly greater hemolysis than the L (curves of type 2).

We also found that physostigmine had little

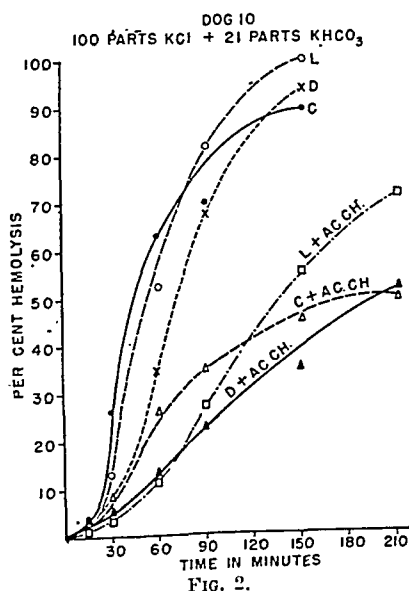


FIG. 2.

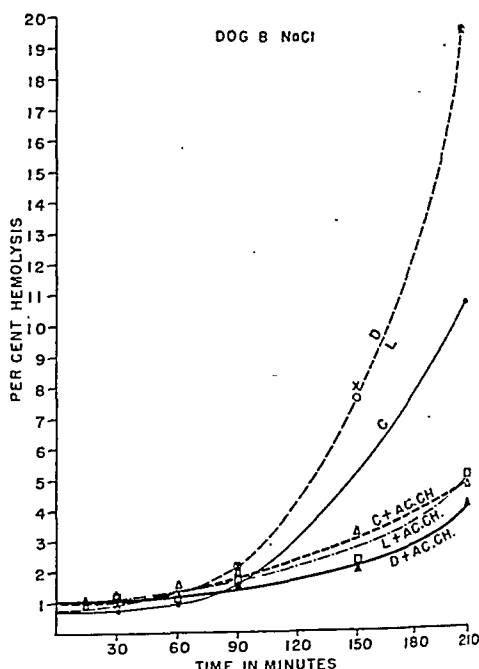


FIG. 3.

effect on permeability in a medium of NaCl as evidenced by hemolysis but that it produced very marked changes in permeability in a high K medium, in the presence of acetylcholine and a pH of 8, under which conditions it produced maximum inhibition of cholinesterase.<sup>1</sup>

Investigations of ionic transfer between erythrocytes and the medium in the presence of cholinesterase inhibitors are under way.

**Summary.** The theory that the acetylcholine-cholinesterase system is concerned with membrane permeability is further substantiated by the finding that the L-isomer of isoamidone which has a greater inhibitory action on cholinesterase than has the D-isomer also changes permeability of dog erythrocytes to a different degree than does the D-isomer.

The conditions under which the L has a greater effect than the D-isomer on permeability are a medium containing a high proportion of K ions relative to Na ions.

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would have increased the incidence and the severity of the kidney lesions.

That vitamin B<sub>12</sub> was the active principle in the concentrates used is indicated by the results shown in Table II. In this series crystalline vitamin B<sub>12</sub> was compared with the charcoal adsorbate at the .04% level of choline chloride.

Vitamin B<sub>12</sub> likewise increased the efficiency of methionine for the prevention of kidney damage (Table III). Supplementary DL-methionine was added to the basal diet at levels of .15% and .192%. The incidence of kidney damage was 100% at both levels. However, when the diet was further supplemented with vitamin B<sub>12</sub>, the incidence of kidney damage decreased to 25% at the .15% level of methionine and to 12% at the .192% level.

The results when vitamin B<sub>12</sub> was added to the diet with an adequate protective level of choline are shown in Table I. There was no effect on weight gains or appearance of the rats at the end of the second or fourth week. Rats in this laboratory have consistently made normal growth on this diet when adequate choline was added without either folic acid or vitamin B<sub>12</sub>. When .2% choline chloride was added to the basal diet the need for supplementary vitamin B<sub>12</sub> was eliminated. It thus appears that dietary choline has a significant sparing action on vitamin B<sub>12</sub>.

**Discussion.** The results of these experiments show that vitamin B<sub>12</sub> decreases the dietary choline or methionine required for protection against the hemorrhagic kidney syndrome in rats. It appears that, under the conditions of these experiments, about one-half of the choline required for protection against this syndrome can be replaced by 30 µg of vitamin B<sub>12</sub> per kg of diet. Moreover, the results of one experiment indicate that the need for vitamin B<sub>12</sub> is markedly decreased by the inclusion of adequate choline in the diet.

Thus, there is established the existence of an interrelationship between vitamin B<sub>12</sub> and choline or methionine when the latter functions as a choline precursor. This interrelationship assumes added significance in view

of the apparent importance of these factors in the maintenance of normal hemoglobin levels in humans as well as in experimental animals. Vitamin B<sub>12</sub> has been shown by West<sup>8</sup> to be effective in the treatment of Addisonian pernicious anemia. Spies and his collaborators<sup>9</sup> have recently reported satisfactory hematologic responses to vitamin B<sub>12</sub> therapy in persons suffering from pernicious anemia, nutritional macrocytic anemia, or tropical sprue. They also found the vitamin to be effective in relieving the subacute combined nervous system degeneration associated with pernicious anemia.

Engel<sup>10</sup> has reported anemia and edema in rats on low-protein diets that were deficient in choline. The addition of choline chloride prevented both the anemia and the edema. McKibbin, Thayer and Stare<sup>10</sup> have reported lowered hemoglobin values in dogs receiving diets deficient in choline. We have observed severe anemia in dogs on a choline-deficient diet similar to the diet used in the experiments herein reported.<sup>11</sup>

Choline has been used for the treatment of anemia in humans with variable results. Moosnick, Schleicher and Peterson<sup>12</sup> have reported successful treatment with choline of a case of pernicious anemia that was refractory to parenteral liver therapy. Davis and Brown<sup>13</sup> have reported hematologic responses to choline therapy in 4 case of pernicious anemia and one case of megaloblastic anemia of pregnancy; choline was ineffective, however, in one other case of pernicious anemia and 4 other cases of megaloblastic anemia.

The demonstration of a specific interrelationship of vitamin B<sub>12</sub> and choline in protecting against the hemorrhagic kidney syn-

<sup>8</sup> West, R., *Science*, 1948, **107**, 398.

<sup>9</sup> Spies, T. D., Suarez, R. M., Lopez, G. G., Milanes, F., Stone, R. E., Toca, R. L., Aramburu, T., and Kartus, S., *J.A.M.A.*, 1949, **139**, 521.

<sup>10</sup> McKibbin, J. M., Thayer, S., and Stare, F. J., *J. Lab. and Clin. Med.*, 1944, **29**, 1109.

<sup>11</sup> Schaefer, A. E., and Salmon, W. D., unpublished data.

<sup>12</sup> Moosnick, F. B., Schleicher, E. M., and Peterson, W. E., *J. Clin. Invest.*, 1945, **24**, 278.

<sup>13</sup> Davis, L. J., and Brown, A., *Blood, J. Hematology*, 1947, **11**, 407.

TABLE I.  
Choline-Sparing Action of Vitamin B<sub>12</sub> for Protection Against Hemorrhagic Kidneys.

Dietary supplement		No. of rats	Mortality	Avg wt gain of survivors g/2 wks	Incidence of renal damage, %
Choline Cl, %	Source of vitamin B <sub>12</sub> , ≅ μg/kg diet				
.04	0	25	24	19	100
.05	0	4	0	36	75
.06	0	16	0	44	69
.20	0	4	0	57 (130)*	0
.02	Conc. No. 1† ≅ 30	4	50	50	75
.03	" " 1 ≅ 60	4	0	38	25
.03	" " 1 ≅ 30	4	25	39	50
.04	" " 1 ≅ 30	21	9.5	55	38
.05	" " 1 ≅ 30	4	0	45	25
.06	" " 1 ≅ 30	10	0	57	20‡
.20	" " 1 ≅ 30	4	0	58 (132)*	0

\* Avg wt gain at 4 weeks.

† Merck & Co., Inc.—charcoal adsorbate No. 8R 5704.

‡ Very slight renal damage in 2 of the 10 rats.

TABLE II.  
Choline-Sparing Action of Vitamin B<sub>12</sub> for Protection Against Hemorrhagic Kidneys.

Dietary supplement		No. of rats	Mortality, %	Avg wt gain of survivors, g/2 wks	Incidence of renal damage, %
Choline Cl, %	Source of vitamin B <sub>12</sub> , ≅ μg/kg diet				
.04	None	4	25	14	100
.04	Crystalline B <sub>12</sub> , 30	4	25	43	50
.04	Conc. No. 1 ≅ 30	4	25	39	50

TABLE III.  
Methionine-Sparing Action of Vitamin B<sub>12</sub> Concentrate for Protection Against Hemorrhagic Kidneys.

Dietary supplement		No. of rats	Mortality, %	Avg wt gain of survivors, g/2 wks	Incidence of renal damage, %
DL-methionine, %	Source of vitamin B <sub>12</sub> , ≅ μg/kg diet				
.150	0	4	75	46	100
.192	0	8	25	27	100
.150	Conc. No. 1 ≅ 30	4	25	57	25
.192	" " 1 ≅ 30	8	0	60	12

incidence of kidney damage was 100% and 69%, respectively. The inclusion of a charcoal adsorbate to supply 30 μg of vitamin B<sub>12</sub> per kg of diet to the above treatments reduced the incidence of kidney damage to 38% and 20%, respectively. It appeared that .03% of choline with vitamin B<sub>12</sub> was as effective in preventing kidney damage as .06% of choline without the vitamin.

In general, the severity of renal damage in the rats receiving vitamin B<sub>12</sub> was relatively mild in comparison with the damage in the control rats not receiving the vitamin. Only

2 of 21 rats receiving .04% of choline with vitamin B<sub>12</sub> concentrate died; the average weight gain of the survivors for the 2-week period was 55 g. Of the 25 rats receiving .04% of choline chloride without vitamin B<sub>12</sub>, 6 died and the average weight gain of the survivors for the 2-week period was only 19 g. The increased gain of the rats receiving the vitamin B<sub>12</sub> preparations was in all probability a direct result of the protective action of the vitamin. It certainly emphasizes the protective effect of the vitamin because in the absence of such protection the increased gain

killed more rapidly at higher temperatures and small groups of thinly spread cells at low temperatures, it seemed not unlikely that one of the common denominators might be a question of oxygen supply and demand.

**Methods.** After shaving and scrubbing, the chosen area of rabbit skin was covered for 2 minutes with a wet pack of tincture of iodine diluted 1:2 in water, and again scrubbed with alcohol. The strips of biopsied skin, either full- or half-skin depth were cut into 1x1 cm pieces. One piece of skin was explanted immediately and the others (usually 8) were stored.

The tissues were stored in two types of containers: a) 13 mm test tubes with just sufficient (0.2 cc) mineral oil or 10% rabbit serum to cover tissues, and also in such tubes filled with 9 cc of these fluids in order to replace the atmosphere, or b) in 6 cc screw cap vials suitable for mailing. These vials were packed approximately  $\frac{3}{4}$  full of washed Pyrex glass wool and a second (removable) plug was added to fill the remaining space. One cubic centimeter of sterile medium moistened the entire surface of the fibers by capillarity, leaving about 4.5 cc of air space, while 5.5 cc fluid completely displaced the atmosphere. Penicillin and streptomycin at 100 units per ml. and sulfadiazine at 20 mg% were at times included in the storage media.

**Tissue culture reagents.** 1. Balanced salt solution (BSS): One liter of concentrated stock stored at room temperature with 4 cc chloroform: NaCl, 80 g; KCl, 4.0 g;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 2.0 g;  $\text{CaCl}_2$ , 1.4 g (dissolved separately);  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ , 0.6 g (1.5 mg of  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ );  $\text{KH}_2\text{PO}_4$ , 0.6 g; glucose, 10.0 g. To this is added 100 cc of phenol red 0.2%. This stock solution was diluted 1:10 ( $\text{Ca} = 5 \text{ mg } \%$ ,  $\text{P} = 2.4 \%$ ) and autoclaved in 20 cc amounts in screw cap Pyrex bottles. One half cubic centimeter of autoclaved 1.4% (isotonic)  $\text{NaHCO}_3$  was added to each bottle and the solution stored in the refrigerator for  $\text{CO}_2$  equilibration to pH 7.6 before final tightening of the caps.

2. *Chicken plasma (CH)* Cock blood was drawn into a 10 cc syringe containing 0.2 cc

of 10% sodium citrate, centrifuged twice and stored in 1 to 2 cc amounts in plain glass tubes at  $-20^\circ\text{C}$ .

3. *Beef embryo juice (EM<sub>50</sub>)*: Three- to 5-inch beef embryos were emulsified in a Waring blender for 1.5 minutes with an equal volume of BSS. The suspension was centrifuged at top speed for 30 minutes and the supernate respun in cotton balltubes.

4. Rabbit serum fresh, or frozen at  $-20^\circ\text{C}$ .

Efficient sterility tests were provided by having a small fluff or ball of cotton in all tubes used for the second centrifugation. Clean separation of the supernate from the sediment was assured, while approximately 99% of the possible contaminating organisms remained below the packed cotton. Brewer's medium was pipetted onto the cotton and the sediment to a depth of approximately 3 inches and the whole incubated at  $31^\circ$  for one week.

**Tissue culture methods.** Both freshly biopsied and stored skin\* were sampled for viability after being cut into 2x2 mm fragments in Brewer's medium, which was pipetted off for sterility control. Twelve of the fragments from each piece of skin were selected at random, 3 being transferred to each of 4 tubes previously coated with one drop of chicken plasma. After orientation of the explants and mixing the three drops of medium (see liquid phase) with the plasma, the tubes were laid horizontally for coagulation. One cubic centimeter of liquid phase (rabbit serum 20%, embryo juice 2%, penicillin and streptomycin 100 units each and sulfadiazine 20 mg %) was added to each tube prior to stoppering and slanting for incubation at  $37^\circ$  for one week.

At the end of one week, data were collected on the proportion of explants showing positive growth and on the area of new growth around the explants. Epithelial and fibrocytic outgrowth was distinguished and each rated on a scale of 1 to 4 plus, 1+ being assigned to explants with sufficient cells to surround only one of the four sides and 4+ to those completely surrounded.

\* The removal of mineral oil from stored tissues by shaking them in several replacements of ethyl ether did not influence their viability.

drome in rats, may suggest that the use of choline as a supplementary aid in the treatment of various types of anemia should be further explored. Some of the clinical results referred to above indicate that it may be of particular value in cases complicated with liver damage. Further investigations on the nutritional implications of this interrelationship are underway in this laboratory.

*Summary.* 1. The incidence and severity of renal injury in weanling rats fed diets low in choline and methionine were markedly decreased by supplementing the diet with a vitamin B<sub>12</sub> concentrate or crystalline vitamin B<sub>12</sub>.

2. Under the conditions of these experiments, 30  $\mu$ g of vitamin B<sub>12</sub> per kg of diet could replace about one-half of the supplementary choline or methionine required for protection against kidney damage.

3. When sub-protective levels of choline were fed, the addition of vitamin B<sub>12</sub> caused a significant increase in weight gain. However, when an adequate protective level of choline was fed, no increase in weight gain was obtained from the addition of the vitamin.

4. The results established the existence of an interrelationship between vitamin B<sub>12</sub>, and choline or methionine.

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### 17131. Relation of Oxygen and Temperature in the Preservation of Tissues by Refrigeration.

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An inquiry has been made into conditions which may influence the viability of 1x1 cm areas of biopsied rabbit skin during refrigeration at 0° and 6-8°C. Since the availability of oxygen, as well as the nature of the storage medium, had an important influence on the preservation of viability at these two temperatures, these relationships may be of interest to those wishing to store or ship tissues for surgical and other purposes.

Tissues separated from the circulation rapidly become anoxic and necrotic at room or body temperatures. This condition may be prevented in uterine or intestinal strips by oxygenation or by chilling.<sup>1,2</sup> The survival of ligated limbs<sup>3</sup> and of the cells in whole em-

bryos<sup>4,5</sup> or organs<sup>5,6</sup> is optimal (among the widely spaced temperatures which have been studied) at 0°. At this temperature respiration is minimal, while oxygen solubility in water is twice that at 30°.

Though Lambert,<sup>7</sup> Carrel,<sup>8</sup> and Hetherington and Craig<sup>5</sup> found 0 to 7° favorable for preserving the small masses of crowded cells in embryonic tissue fragments, there is considerable evidence that the thin perimeter of migrating and dividing cells in established tissue cultures has maximal longevity around 30°<sup>9,10</sup> and are unable to re-establish growth after refrigeration for a few days.<sup>9,11-13</sup> Upon considering the fact that large tissues are

<sup>6</sup> Lewis, W. H., and McCoy, C. C., *Bull. Johns Hop. Hosp.*, 1922, **33**, 284.

<sup>7</sup> Lambert, R. A., *J. Exp. Med.*, 1913, **18**, 406.

<sup>8</sup> Carrel, A., *J. Amer. Med. Assn.*, 1912, **50**, 523.

<sup>9</sup> Nemoto, M., *Tohoku J. Exp. Med.*, 1929, **14**, 1.

<sup>10</sup> Hanks, J. H., *J. Cell. and Comp. Physiol.*, 1948, **31**, 235.

<sup>11</sup> Carpenter, E., *J. Exp. Zool.*, 1945, **98**, 79.

<sup>12</sup> Fischer, A., *Arch. f. exp. Zellf.*, 1926, **2**, 303.

<sup>13</sup> Hanks, J. H., unpublished.

<sup>1</sup> Garry, R. C., *J. Physiol.*, 1928, **66**, 235.

<sup>2</sup> Nolf, P., *Arch. Internat. de physiol.*, 1928, **30**, 315.

<sup>3</sup> Allen, F. M., *Surg., Gynec. and Obst.*, 1938, **67**, 746.

<sup>4</sup> Bucciantie, L., *Arch. f. exp. Zellf.*, 1931, **11**, 397.

<sup>5</sup> Hetherington, D. C., and Craig, J. S., *J. Cell. and Comp. Physiol.*, 1939, **14**, 197.

*Results.* Representative results are summarized in Table I. During refrigeration at 6-8°, the exclusion of atmosphere was always detrimental (see first experiment). In tubes or vials filled with mineral oil the blood in the vessels became blue, and the viability declined in less than one week. No cells could be cultivated after two weeks. In tubes filled with rabbit serum 10% the viability declined steadily over the two week period. The pH indicator in the upper stratum of the liquid retained its original color while the fluid nearer the tissues became strongly acidified within one week.

With just enough oil to cover the skin and prevent evaporation, the blood in the vessels remained red. Viability was maintained at a high level for one week, but declined sharply during the second week. In shallow layers of rabbit serum 10% the pH fell to 6.8 or 7.0 within one week but in spite of the small reserve of medium and of buffer the viability did not decline within two weeks.

Preservation of viability at 0° proved to be far less satisfactory in any medium employed, and particularly in the presence of atmosphere (see second experiment). No biopsies survived for one week in 0.2 to 1.0 cc mineral oil. In tubes filled with mineral oil to exclude atmosphere, the results were strikingly more favorable, since approximately 50% of the cells remained alive during the first week. No cells survived after preservation in oil for two weeks. In rabbit serum 10% of the cells were more adequately supported or protected, and the results again revealed the same relationship with respect to the influence of atmosphere. In the shallower layers of medium the viability at the end of one or two weeks was only 50 to 70% of that in tubes which had been completely filled with liquid.

Since blood provides a mass of cells capable of competing for the available oxygen, this menstruum was also employed in certain of the experiments at 0°. The third experiment includes data on both Tiersch graft and full-depth biopsies removed from the same rabbit and stored at 0° for one week. It may be seen that a blood coagulum of only one centimeter in depth proved as effective as completely filling the tubes with rabbit serum

10% or 100%. Greater depths of blood (e.g. 3 cm) were not superior, but were sometimes disadvantageous.

The poor migration of epithelial cells in certain experiments was due to incomplete removal of the iodine used for skin sterilization (see next paper).

*Discussion and summary.* Although the storage of tissues at low temperature has long been recognized as a convenient means of preserving viability for relatively short periods of time, the potential advantages of refrigeration require further investigation of the nutrient materials needed to sustain metabolism and of the conditions which permit conservation of the essential enzyme systems. From the present comparison of survival in mineral oil and in dilute serum, it is evident that the usefulness of vaseline,<sup>8,14</sup> pliofilm<sup>15</sup> or moist gauze<sup>16</sup> as storage vehicles may be questioned. These earlier methods provide no nutrient materials and make no provision for leaching out or buffering the acids which are produced in the living tissues.

No simple rules can be stated for the control of the oxygen-temperature relationship, since it will be modified by the depth of the medium employed and by the form and mass of the tissue, and must be determined for each type and volume of tissue to be stored. For the shipping of tissues in iced containers the use of a blood coagulum may be regarded on the one hand as a convenient means of increasing the cell mass or, on the other, of providing the necessary exclusion of atmospheric oxygen.

Since blood does not permit observation of pH changes as an index of the need to replace nutrient fluids, and the blood cells must inevitably contribute to acidification and exhaustion of the medium, this menstruum is considered less desirable than a non-cellular fluid for the "banking" of tissues during hospital storage for surgical purposes. Furthermore, it is evident that during refrigeration at 6-8° any increases in nutrient and buffering

<sup>14</sup> deMartigny, F., *Congr. franc. chir.*, 1913, **26**, 252.

<sup>15</sup> Webster, J. P., *Am. J. Surg.*, 1944, **120**, 431.

<sup>16</sup> Matthews, D. N., *Lancet*, 1945, **1**, 775.



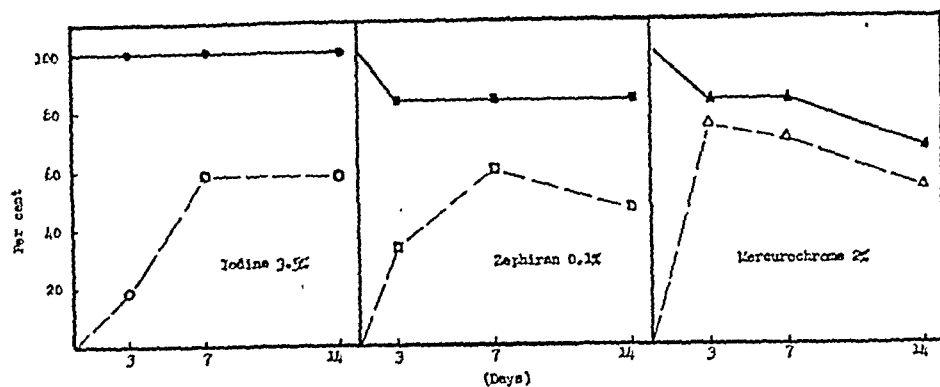


FIG. 1.

The proportion of explants free of contamination (solid symbols) and the proportion positive for epithelial cells (open symbols). Thirty-three cultures in each group.

curochrome. The results (illustrated in Fig. 1) were the usual ones after iodine patching, namely, no contamination, but appreciable inhibition of epithelial migration. With Zephiran 17% of the cultures were lost by contamination within the first 3 days, while migration of epithelium was not significantly improved. The Mercurochrome treatment was definitely less inhibitory to epithelial cells, but did not afford an increase in the number of useful cultures, due to a 33% loss by contamination. Since fibroblasts grew from the skin with equal readiness irrespective of the disinfecting agent, the dilute iodine produced the greatest proportion of positive and usable cell cultures.

Lest the aqueous solution of Mercurochrome might have given results poorer than those obtainable in the presence of a wetting agent, the effectiveness of Mercurochrome 2% in Duponol was compared against tincture of iodine diluted 1:4 in 70% alcohol, each being applied as a moist pack for 2 minutes prior to the biopsy of rat skin. During the first two weeks of cell cultivation *in vitro*, the incidence of contamination proved to be 20% from the Mercurochrome biopsy and 4% following the use of the 1:4 dilution of iodine.

Subsequent work with tincture of iodine 1:4 in 70% alcohol, with application for five minutes, indicates complete reliability, but that the same care must be exercised in its removal as in the case of the 1:2 solution.

Further experience is required to evaluate the routine effectiveness of the 1:4 dilution during application for only two minutes.

**Discussion.** In Price's excellent report on disinfection of skin,<sup>1</sup> it is emphasized that the germicidal action of 7% iodine tincture practically ceases upon drying. It is shown that 2% of iodine in alcohol 70% by weight evaporates more slowly and avoids the objectionable irritating effects of the 7% tincture. This 2% solution was more effective following a single surface application than any other class of disinfectant studied. In other sections of the report the time factor is repeatedly emphasized. The results reported represent an application of the principles elucidated by Price.

It is not improbable that a mixture of penicillin, streptomycin and sulfadiazine for skin treatment, and in the cell culture media would provide a more appropriate measure of optimal epithelial migration than any of the disinfectants used. In view of the long period of cell cultivation required in leprosy research, this comparison has been impractical for two reasons: (a) the danger of inhibiting the infectious agent, and (b) the inability of such a mixture to inhibit the development of fungi from the skin explants.

<sup>1</sup> Price, P. B., *J. A. M. A.*, 1938, **111**, 1939.



fluids should be made horizontally, *i.e.*, without restricting the availability of atmospheric oxygen.

A study by the surgical staff of the Children's Hospital of the preservation of aortic segments for surgical repair of large blood vessels has produced results in accordance with these principles. Their publication<sup>17</sup> describes the conditions under which blood

vessels may be maintained in 10% homologous serum for as long as 50 days.

In conclusion, 10% serum is superior to mineral oil as a refrigeration menstruum because it provides nutrient and dilutes or buffers the acids which result from metabolism. At 6-8° oxygen is required to support metabolism, while at 0° viability is extended by completely filling the storage tubes with liquid or by covering the tissue with a blood coagulum.

<sup>17</sup> Peirce, E. C., Gross, R. E., Bill, A. H., and Merrill, K., *Ann. Surg.*, 1949, **129**, 333.

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### 17132. Sterilization of Skin.

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It is frequently stated that in the strict bacteriological sense it is impossible to sterilize skin. This concept apparently arises from the use of methods which do not insure that an appropriate disinfectant is applied in active form for an adequate interval of time. Since the rich and complex nutrients used for cell cultivation provide an excellent pabulum for a variety of microorganisms, long experience in sterilization of leprous and normal skin prior to biopsy for tissue culture induces us to present evidence that skin sterilization is not a difficult problem.

Early attempts to sterilize well scrubbed skin by painting with tincture of iodine were at times successful and at times failures. The inconstancy of the results suggested the desirability of controlling the action of iodine with respect to concentration and time of action. By covering the area to be biopsied with a heavy patch of fabric, dripping iodine solution (tincture of iodine 1:2 in 70% alcohol by weight) on this patch until it was saturated, and then removing the patch and recleaning with alcohol after an interval of 5 minutes, the occurrence of scattered contamination was terminated. During the next several years it was noted that epithelial cells

rarely if ever migrated from fragments explanted from the papillary or uppermost layer of the skin, while abundant epithelial cells appeared around fragments explanted from the deeper or reticular layer of the skin.

Upon the first occasion when a biopsy was taken after painting the skin with 2% Mercurochrome in Duponol as a wetting agent, good epithelial growth around papillary explants appeared for the first time in our experience. Nevertheless, one after another of the culture tubes gave evidence of contamination by staphylococci until 40% of the cultures had been lost. The period of applying the iodine (1:2) patch was next shortened to 2 minutes, without trouble from contamination and with the result that the superficial layer of skin produced moderate to excellent epithelial migration.

In order to inquire whether other disinfectants might be as effective as dilute iodine under these conditions and possibly less inhibitory to epithelial migration *in vitro*, three strips of skin were removed from the same individual simultaneously, one having been prepared by a two minute dressing with tincture of iodine 1:2, the second with Zephiran 1:1000, and the third with 2% aqueous Mer-

TABLE I.

Exp. No.	Dietary supplement		No. of chicks	Avg wt gain g/chick	
	Choline chloride %	Source of vitamin B <sub>12</sub> $\mu$ g B <sub>12</sub> /kg diet		2 wk	4 wk
4	.05	0	11	20	46
3, 4	.1	0	23	27	60
2	.2	0	12	31	95
1, 2, 3, 4	.6	0	47	54	154
4	.05	B <sub>12</sub> conc. No. 1 $\approx$ 15	11	41	150
3	.1	" " " 1 $\approx$ 30	12	90	246
4	.1	" " " 1 $\approx$ 15	11	72	262
1, 2, 3	.2	" " " 1 $\approx$ 30	34	63	195
1, 2, 3, 4	.6	" " " 1 and 3 $\approx$ 30	59	79	218
1, 3	.6	" " " 1 and 3 $\approx$ 15	22	78	210

diet was supplemented with .6% choline chloride plus vitamin B<sub>12</sub> concentrate (Fig. 1).

When the choline supplement was .05%, the increase in weight resulting from the addition of vitamin B<sub>12</sub> concentrate was less than that observed in the groups receiving .1% and .2% choline chloride. This indicates that this level of vitamin B<sub>12</sub> cannot replace the entire dietary choline required by the chick but that a minimum level of choline must be present.

That vitamin B<sub>12</sub> was the active principle in the concentrates is indicated by the data in Table II. Pure vitamin B<sub>12</sub> fed at a level of 15  $\mu$ g per kg of diet caused an average increase in weight gain of 14 g at 1 week and 40 g in 2 weeks as compared to 14 g at 1 week and 45 g in 2 weeks for the group receiving the vitamin B<sub>12</sub> concentrate.

**Discussion.** The choline requirement for normal growth of chicks fed the basal diet without a source of vitamin B<sub>12</sub> was .6% choline chloride as compared to .1% when vitamin B<sub>12</sub> was added to the diet. This in-

terrelationship of vitamin B<sub>12</sub> and choline for the chick is in agreement with the results obtained with rats.<sup>1</sup>

The chicks used in these experiments were progeny of hens from a strain that had been bred for high livability. The breeding flock is on commercial breeding ration, which supports good egg production. The progeny of these hens have shown a very uniform growth response to the addition of vitamin B<sub>12</sub>. Not only was this growth increment uniform but it was also much greater than that reported by other investigators using other types of diets. For example, when the basal diet was supplemented with .1% choline chloride plus a source of vitamin B<sub>12</sub>  $\approx$  30  $\mu$ g per kg of diet, the growth increment was 63 g at 2 weeks and 186 g at 4 weeks. This increment is considerably greater than that reported by the following workers: Lillie, *et al.*,<sup>2</sup> 34 to 60.3 g increase in body weight from the second to the fourth week; Nichol, *et al.*,<sup>6</sup> 79 to 88 g for a similar period, and Ott, *et al.*<sup>7</sup> of 20 to 46 g at 17 days.

These observations of the influence of choline level on the vitamin B<sub>12</sub> requirement may be useful when procedures for a chick assay of vitamin B<sub>12</sub> are being standardized. The data presented in Fig. 1 indicate that vitamin B<sub>12</sub> can be assayed more effectively if

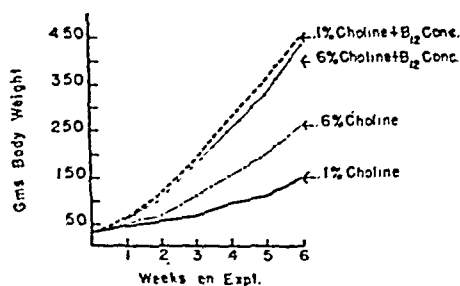


FIG. 1.

Sparing action of vitamin B<sub>12</sub> concentrate on the choline requirement of chicks. (12 chicks per group).

<sup>2</sup> Lillie, R. J., Denton, C. A., and Bird, H. R., *J. Biol. Chem.*, 1948, **170**, 1477.

<sup>6</sup> Nichol, C. A., Dietrich, L. S., Cravens, W. W., and Elvehjem, C. A., *Proc. Soc. Exp. Biol. and Med.*, 1949, **70**, 40.

<sup>7</sup> Ott, W. H., Riekes, E. L., and Wood, T. R., *J. Biol. Chem.*, 1948, **174**, 1047.

# 17133. Interrelationship of Vitamin B<sub>12</sub> and Choline. II. Effect on Growth of the Chick.\*

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In the accompanying paper it was reported that the severity and incidence of renal hemorrhage in weanling rats fed diets low in choline and methionine were significantly decreased by supplementing the diet with vitamin B<sub>12</sub>.<sup>1</sup> In concurrent experiments the interrelationship of the vitamin B<sub>12</sub> and choline requirements for growth of the chick was studied.

**Experimental.** Day-old Leghorn chicks of the A.P.I. strain were divided into 3 weight classes ranging from 34 to 42 g. The selected chicks were then distributed, 8 to 12 per group, so that the group average was about 38 g. The chicks were wing-banded and housed in standard commercial, electrically heated batteries, which were equipped with raised wire floors. Feed and water were supplied *ad libitum* and the chicks were weighed weekly. The basal diet was composed of extracted peanut meal 30,<sup>2</sup> sucrose 36.9, extracted casein 6,<sup>3</sup> L-cystine .1, cod liver oil 1, lard 19, Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub> 2.87, K<sub>2</sub>HPO<sub>4</sub> 1.442, CaCO<sub>3</sub> .903, MgSO<sub>4</sub> · 7H<sub>2</sub>O .840, NaCl .70, Fe Citrate .182, MnSO<sub>4</sub> · 4H<sub>2</sub>O .035, ZnCO<sub>3</sub> .014, CuSO<sub>4</sub> · 5H<sub>2</sub>O .007, and KI .007. Vitamin supplements, mg/kg diet, were as follows: thiamine 3, pyridoxine 4, riboflavin 6, calcium

pantothenate 20, niacin 50, inositol 1000, folic acid 1, biotin .25, alpha tocopherol 25, alpha tocopherol acetate 25, and menadione 5. The diets were mixed at 10-day intervals and stored at 1°-5°C. The basal diet furnished a maximum of .007% choline and .3% methionine. Preliminary studies had established that supplementation of this basal diet with .6% choline chloride was necessary for maximum growth.

A summary of four experiments is given in Table I. Concentrates of vitamin B<sub>12</sub> were added to the basal diet at the expense of sucrose and the choline supplement was reduced in the amount that was furnished by the concentrates. Choline was determined by the procedure of Engel,<sup>4</sup> modified by washing the choline reineckate precipitate with cold N-propyl alcohol in place of methyl alcohol. Vitamin B<sub>12</sub> concentrates Nos. 1 and 3 were a charcoal and fullers earth adsorbate, respectively. The crystalline vitamin B<sub>12</sub> was in the form of a solution containing 25 µg/ml.

When the basal diet was supplemented with .6% choline chloride, the addition of vitamin B<sub>12</sub> concentrates Nos. 1 or 3 at a level to supply 30 µg vitamin B<sub>12</sub> per kg diet caused an average increase in weight gain of 24 to 28 g in 2 weeks, or 59 to 66 g in 4 weeks. The feeding of the concentrates to supply 15 µg vitamin B<sub>12</sub> per kg diet resulted in a weight increment of 52 to 62 g in 4 weeks.

When the choline supplement was reduced to .2%, the addition of vitamin B<sub>12</sub> concentrate caused an increase in weight gain at 2 weeks of 32 g and 100 g in 4 weeks. At the .1% level of choline chloride supplement the greatest increase in weight gain due to the vitamin B<sub>12</sub> concentrate was observed. The weight increment was 63 g at 2 weeks and 186 g at 4 weeks. The total weight of these birds was as good as that obtained when the

\* Published with the approval of the Director, Alabama Agricultural Experiment Station. This study was supported in part by a grant from the American Cancer Society upon recommendation of the Committee on Growth, National Research Council. It was aided by donations of vitamin B<sub>12</sub> concentrates, crystalline vitamin B<sub>12</sub>, and other vitamins by Merck & Company, and of folic acid by Lederle Laboratories. These data were presented in part at the Detroit meeting, Fed. Am. Soc. Exp. Biol., April 20, 1949.

<sup>1</sup> Schaefer, A. E., Salmon, W. D., and Strength, D. R., *Proc. Soc. Exp. Biol. and Med.*, 1949, **71**, 193.

<sup>2</sup> Engel, R. W., *J. Nutrition*, 1948, **36**, 739.

<sup>3</sup> Salmon, W. D., *J. Nutrition*, 1947, **33**, 155.

<sup>4</sup> Engel, R. W., *J. Biol. Chem.*, 1942, **144**, 701.

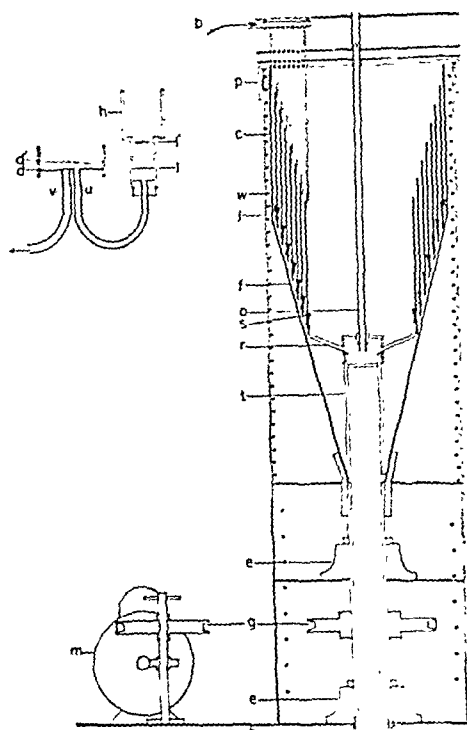


FIG. 1.

Schematic representation of oxygenator. b, blood inlet; c, cylinders upon which blood films; d, rubber dam covering outlet; d', rubber dam raised to permit flow; e, bearing; f, blood collecting funnel; g, pulleys for revolving oxygenator; h, collecting cup; j, insulated jacket; l, level of blood when rubber dam shuts off blood flow; l', level of blood when rubber dam is raised; m, motor; o, oxygen inlet tube; p, plastic cover; r, rack which holds concentric cylinders and conducts oxygen to the cylinders; s, spouts which deliver oxygen to each cylinder; t, hollow shaft which delivers blood to cup; u, blood inlet tube to level control; v, blood outlet tube from level control to pump; w, warm water coil.

6% CO<sub>2</sub> gas mixture is piped down into the shaft (o) and through the hollow tubing of the rack (r) which supports the cylinders. There are four oxygen inlets (s) supplying each cylinder. The shaft, cone, and cylinders all rotate as one unit at 150 r.p.m. There is very little foaming of blood.

The blood is pumped from the animal and back to the animal by pumps which have an intermittent pulsatile action. Ball valves give directional flow. Steady flow into the oxygenator is obtained by having 3 such pumps in a parallel arrangement.

The blood level in the collecting cup is maintained by a leveling device which consists of a plate with a sheet of rubber dam (d) stretched over it. A hole in the plate admits blood from the collecting cup, another allows the blood to flow into the "arterial" pump. The level of the plate in relation to the cup determines the level of blood in the cup because hydrostatic pressure of blood in the cup raises the rubber dam (d') off the plate and allows blood to flow under the dam to the pump. When the level of blood in the cup is at the level of the plate (1) the rubber dam is sucked down onto the plate by the pump and no blood flows. Because the action of the pump is intermittent, the dam is again separated from the plate by the blood when the level in the cup rises (1'). Body temperature is maintained with warm water coils (w) around the oxygenator and warm air around the collecting cup and leveling device. A filter with 230 micra spaces is used in the "arterial" stream.

To date 13 perfusions of dogs have been done. In 4 instances blood was withdrawn from the femoral veins, and in 9 the vena cava was cannulated. Blood was infused via the femoral, brachiocephalic, or the subclavian artery. Blood flow rates were estimated with a venturimeter. The volume of the film was measured by turning off the pumps to the oxygenator and allowing the blood on the cylinders to collect in a graduate. Oxygen content of blood as it left the animal (venous) and as it returned (arterial) was determined by the Van Slyke method.<sup>4</sup>

**Results.** The results of 5 experiments in which data were collected are tabulated in Table I. At the flow rates obtained by pumping all available blood from the vena cava a film could be maintained on only 4 or 5 cylinders. Thus, the total capacity of the oxygenator is considerably greater than that utilized in these instances. It was noted that the animals made very few slight respiratory movements during perfusion even though no pulmonary respiration was maintained. The efficiency of the oxygenator (no. of cc O<sub>2</sub>

<sup>4</sup> Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, 1924, 61, 523.

TABLE II.  
Crystalline Vitamin B<sub>12</sub> Compared with Vitamin B<sub>12</sub> Concentrate.

Dietary supplement			Avg wt gain g/chick	
Choline chloride %	Source of vitamin B <sub>12</sub> ≅ μg per kg diet	No. of chicks	7 days	14 days
.1	None	11	16	27
.1	Crystalline vit. B <sub>12</sub> ≅ 15	7	30	67
.1	B <sub>12</sub> conc. No. 1 ≅ 15	11	30	72

the basal diet is supplemented with .1% choline chloride than when it is supplemented with .6%. It would be of interest to determine whether the feeding of a low-choline diet to hens would increase the requirement for the "animal protein factor" in the progeny, similar to that noted when the hens were restricted to a diet low in the "animal protein factor".<sup>5,6</sup>

The interesting interrelationship of vitamin B<sub>12</sub>, choline, and methionine is being studied further in this laboratory.

**Summary.** 1. Vit. B<sub>12</sub> was fed to Leghorn chicks receiving a choline-low basal diet supplemented with .6%, .2%, .1%, and .05% choline chloride. The 14-day increment in

weight gain over the controls that did not receive vitamin B<sub>12</sub> was 26 g, 32 g, 63 g, and 21 g, respectively.

2. Crystalline vit. B<sub>12</sub> fed at a level of 15 μg per kg of diet and at a choline level of .1% was as effective as vit. B<sub>12</sub> concentrate in a 2-week feeding period.

3. The choline requirement of chicks under the conditions of these experiments was markedly reduced by the supplementation of the diet with vit. B<sub>12</sub>.

4. It appears that dietary choline has a significant sparing action on vit. B<sub>12</sub>.

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### 17134. An Oxygenator with Increased Capacity: Multiple Vertical Revolving Cylinders.\*

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The vertical revolving cylinder has been shown to be an effective oxygenator for blood.<sup>1,2</sup> Its efficiency as an oxygenator is probably increased by making the cylinder shorter in relation to its diameter.<sup>3</sup> These data were applied in the construction of an-

other oxygenator with approximately eight times the capacity of the oxygenator previously studied.

**Experimental.** Eight concentric cylinders (Fig. 1, C) 21 cm in height, ranging from 27.5 cm to 17.0 cm in diameter are mounted so that the lower edges of the cylinders are approximately one centimeter above the inner surface of a cone (f). The surface area is 14,500 cm<sup>2</sup>. Blood is delivered through individual spouts (b) onto the inner surface of each cylinder, drops off of its lower edge onto the inner surface of the cone, flows into the vertical shaft (t), runs down the inner surface of the shaft, and collected in the stationary cup (h) beneath. A 94% O<sub>2</sub> and

\* Aided by a grant from the United States Public Health Service, and by a research grant from the Graduate School, University of Minnesota.

<sup>1</sup> Gibbon, J. H., Jr., *J. Lab. and Clin. Med.*, 1939, **24**, 1192.

<sup>2</sup> Gibbon, J. H., Jr., and Kraul, C. W., *J. Lab. and Clin. Med.*, 1941, **26**, 1803.

<sup>3</sup> Karlson, K. E., Dennis, C., and Westover, D. E., *Proc. Soc. Exp. Biol. and Med.*, 1949, **70**, 225.

TABLE I.  
Hemagglutination of Different Species by Normal and Poliomyelitis Stool Suspensions.

Stool No.	Final agglutination titer				
	Human group O	Chick	Rabbit	Guinea pig	Sheep
Poliomyelitis stools.					
7	1:30	1:30	1:20	1:20	1:20
16	1:20	1:40	1:160+	1:20	1:20
17	1:320+	1:320+	1:320+	1:60	1:320+
18	1:120	1:120	1:320+	1:40	1:80
Normal stools.					
3	1:20	1:30	1:320+	1:40	1:20
4	1:40	1:40	1:160	1:20	1:20
5	1:20	1:20	1:320+	1:20	1:20
6	1:120	1:120	1:320+	1:40	1:80

TABLE II.  
Inhibition of Stool Hemagglutination by Serum and Bovine Albumin Using Chick Cells.

Exp.	Serum	Serum dilution								Titer
		1:6	1:12	1:24	1:48	1:96	1:192	1:384	1:768	
1	Human	0	0	0	0	±	+	+	+	1:48+
2	Rabbit	0	0	0	0	+	+	+	+	1:48
3	Monkey	0	0	0	0	0	0	0	±	1:384+
4	20% bovine albumin	0	0	0	+	+	—	—	—	1:24

0 = No agglutination.

+ = Agglutination.

*Methods.* 10% stool suspensions were prepared by grinding the stools in a mortar and pestle with a small amount of physiological saline, diluting the suspensions to a final concentration of 10% and centrifuging in an angle centrifuge at 5000 r.p.m. for 20 minutes. The supernatant was then removed and cultured for the presence of bacteria. Titrations were carried out by making serial 2-fold dilutions of stool suspension in saline. 0.5 cc of material was added to each test tube. Next equal parts of 0.75% of the various erythrocyte suspensions which had been previously washed 3 times prior to use were added. The tubes were mixed and allowed to stand at room temperature for 1 hour prior to reading. The titer was read as the highest dilution showing a hemagglutination pattern on the bottom of the test tube. This pattern closely resembled that produced by influenza and other viruses.

*Results.* Titrations obtained comparing poliomyelitis and normal stool suspensions with group "O" human, chick, rabbit, guinea pig and sheep erythrocytes are shown in Table I. Highest titers were obtained with

rabbit erythrocytes while titers with the erythrocytes of other species did not differ significantly. It was found that rabbit cells in saline occasionally showed spontaneous agglutination and such experiments were discarded. The results summarized in Table I indicate that there was no significant difference in hemagglutination titers between normal and poliomyelitis stool suspensions. In other experiments it was found that a 10% mouse brain and cord suspension infected with the Lansing strain of poliomyelitis virus failed to agglutinate chick, human group "O" and rabbit cells.

Table II shows the inhibition of hemagglutination of chick erythrocytes by human, rabbit, and monkey serum and 20% bovine albumin in relatively high dilutions. Salk's technic<sup>3</sup> of the hemagglutination-inhibition test for antibodies against the influenza virus was employed. Similar results were obtained in hemagglutination-inhibition tests with the erythrocytes of other species. The effect of heat on hemagglutination of chick cells by

<sup>3</sup> Salk, J. E., *J. Immunol.*, 1944, 49, 87.

TABLE I.  
Results of Five Perfusions Withdrawing Blood from the Vena Cava and Returning It Through a Femoral Artery.

Length of perfusion (min.)	Blood flow (cc/min.)	Film vol. (cc)	No. of cylinders filmed	ccO <sub>2</sub> introd. per cc film per min.	ccO <sub>2</sub> introd. per min.	Survival time	Plasma hemoglobin (g%)	
							Before	After
10½	720	300	4	.272	81.7	6 wk*		
12	680	270	5	.272	73.4	1½ hr	.57	.54
11	840	210	5	.284	59.6	10 min.	.25	.23
21	760	200	4	.266	53.2	1½-hr	.70	.71
22	1250	265	6	.295	78.3	3 hr	.80	.94

\* Alive.

introduced into the blood per cc of film per minute) is seen to compare favorably with that in earlier experiments (0.247 cc). There was one other survival. The remainder expired at periods of 30 minutes to 36 hours after perfusion. Hemolysis was found to increase very little during perfusion.

*Conclusions.* 1. An oxygenator consisting of 8 concentric revolving cylinders which utilizes

a funnel to deliver the blood into a small cup is described.

2. Animal perfusions demonstrate that its efficiency as an oxygenator is high enough to make it possible to perfuse the entire animal body without using an impractically large amount of blood in the extra-corporeal circulation.

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### 17135. Hemagglutination by Normal and Poliomyelitis Stool Suspensions.\*

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The phenomenon of agglutination of erythrocytes by certain viruses is well known.<sup>1</sup> Viruses known to produce hemagglutination include influenza A & B, vaccinia, ectromelia, mumps, mouse pneumonitis, fowl plague and Newcastle disease. These agglutination reactions can be differentiated from each other by inhibition with specific immune sera. Certain bacteria and pleuropneumonia organisms also have the property of agglutinating red blood cells. Recently, the presence of a hemagglutinin in bacteria-free amniotic fluid of normal embryonated hens eggs has been described by the Army Commission on Acute Respiratory Diseases.<sup>2</sup> This substance was shown to be associated with the globulin frac-

tion of egg albumin and gains access to amniotic fluid when the contents of the albumin sac ruptures into the amniotic cavity between the 11th and 13th day of incubation. Hemagglutination can also be induced by ricin, abrin, croton and certain inorganic colloidal acids or bases such as salicylic acid and basic proteins as protamine.

In studies on the detection of possible hemagglutination by the poliomyelitis virus, it was found that both normal and infected saline stool suspensions agglutinated the erythrocytes of a wide variety of animal species. This paper is a report describing the agglutination of group "O" human, chick, rabbit, guinea pig and sheep erythrocytes by stool suspensions and some of the properties of the stool hemagglutinin.

\* Supported in part by the Michael Reese Research Foundation.

<sup>1</sup> Rivers, T. M., *Viral and Rickettsial Infections of Man*, J. B. Lippincott Co., 1948, p. 77.

<sup>2</sup> Commission on Acute Respiratory Diseases, *Proc. Soc. Exp. Biol. and Med.*, 1946, 62, 118.

reported by Griffiths.<sup>5</sup> This worker found that *E. coli*, *Acrobacter aerogenes*, *Alcaligenes faecalis*, *Bacillus anthracis*, *Brucella abortus*, *Corynebacterium diphtheriae*, *Eberthella typhosa*, *Proteus vulgaris*, *Salmonella*, *Shigella*, *Staphylococcus aureus* and a few other bacterial species failed to agglutinate human erythrocytes. On the other hand, 23 strains of *Shigella alkalescens*, some strains of *Hemophilus pectusis*, *Vibrio comma*, and *Streptococcus pyogenes* showed hemagglutination of human erythrocytes. None of the species of bacteria which Griffiths reported to produce hemagglutination were isolated from stool suspensions cultured in the present study.

**Summary.** Saline suspensions of stools obtained from both normal and poliomyelitis patients showed hemagglutination of group "O" human, chick, rabbit, guinea pig and sheep

erythrocytes. The rabbit cells were agglutinated in highest titers. No significant difference in hemagglutination titer was noted with the normal and poliomyelitis stools. Normal human, rabbit and monkey serum and 20% bovine albumin inhibited hemagglutination. The hemagglutinin was thermolabile and absorbed by group "O" human, chick and rabbit erythrocytes. Stool suspension Seitz filtrates failed to show hemagglutination, but the titer could be partially restored by methanol precipitation. Various species of bacteria isolated from stool suspensions failed to agglutinate chick, group "O" human and guinea pig erythrocytes but showed some agglutination of rabbit cells in low titer. Mouse brain and cord suspensions infected with the Lansing strain of poliomyelitis virus also failed to agglutinate chick, human group "O" and rabbit erythrocytes.

<sup>5</sup> Griffiths, J. J., *Proc. Soc. Exp. Biol. and Med.*, 1948, **67**, 358.

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### 17136. An Antithyrototoxic Factor for the Rat Not Identical with Vitamin B<sub>12</sub>.\*

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It has been demonstrated that whole liver powder will prolong survival and counteract growth retardation of immature rats fed massive doses of desiccated thyroid.<sup>1-3</sup> Some con-

fusion exists, however, regarding the nature of the factor or factors responsible for the above effects. On rations containing soybean meal the retardation in growth caused by massive doses of desiccated thyroid or iodinated casein has been counteracted both in the rat and chick by liver fractions with vitamin B<sub>12</sub> activity.<sup>4-6</sup> In subsequent work similar findings were obtained with crystalline vitamin

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Communication No. 203 from the Department of Biochemistry and Nutrition, University of Southern California.

<sup>1</sup> Ershoff, B. H., *Proc. Soc. Exp. Biol. and Med.*, 1947, **64**, 500.

<sup>2</sup> Ershoff, B. H., *Arch. Biochem.*, 1947, **15**, 365.

<sup>3</sup> Bethell, J. J., Wiebelhaus, V. D., and Lardy, H. A., *J. Nutrition*, 1947, **34**, 431.

<sup>4</sup> Bosshardt, D. K., Paul, W. J., O'Doherty, K., Huff, J. W., and Barnes, R. H., *J. Nutrition*, 1949, **37**, 21.

<sup>5</sup> Register, U. D., Ruegamer, W. R., and Elvehjem, C. A., *J. Biol. Chem.*, 1949, **177**, 129.

<sup>6</sup> Nichol, C. A., Robblee, A. R., Cravens, W. W., and Elvehjem, C. A., *J. Biol. Chem.*, 1949, **177**, 631.



TABLE III.  
Effect of Heat on Hemagglutination by Stool Suspensions of Chick Cells.

Stool No.	Temperature °C	Time	Titer
3	Unheated 65	— 15 min.	1:30 0
7	Unheated 56 65	— 30 min. 15 min.	1:400 1:60 0
17	Unheated 65	— 15 min.	1:120 0
18	Unheated 65	— 15 min.	1:40 0

TABLE IV.  
Hemagglutination of a Stool Suspension after Previous Absorption with Human Group O, Chick and Rabbit Erythrocytes.

Absorbing cells	Final agglutination titer			
	Human group O	Chick	Guinea pig	Rabbit
Group O	1:10	1:20	1:20	1:240?
Rabbit cells	1:60	1:20	1:20	1:20
Chick cells	1:20	1:20	—	—
None	1:240	1:240	1:60	1:320+

stool suspensions is shown in Table III. Incubation in a water bath at 65°C for 15 minutes completely destroyed the stool hemagglutinin in the experiments listed in Table III, while heating at 56°C for 30 minutes also reduced the titer significantly. In other experiments, the hemagglutination titer was approximately the same when carried out at 3-5°C, room temperature and 37°C. Storage for 2 weeks in the icebox resulted in a marked drop in titer. Stool suspension Seitz filtrates failed to show hemagglutination, but titers could be partially restored ( $\frac{1}{4}$  to  $\frac{1}{2}$  the original) by methanol concentration at low temperatures.<sup>4</sup>

The hemagglutinin could be absorbed in differential erythrocyte absorption experiments by group "O" human, chick and rabbit erythrocytes (Table IV). The absorption was carried out by adding equal parts of 50% packed cells to the stool suspension, incubating for 15 minutes at room temperature and centrifuging at 1500 r.p.m. for 15 minutes. The supernatant was then drawn off and tested for the presence of hemagglutinins

against homologous and heterologous erythrocytes. Usually a single absorption was adequate for almost complete removal of the hemagglutinins.

*Discussion.* The relationship of the stool hemagglutinins to those produced by viruses, bacteria and normal amniotic fluid is unknown. The stool hemagglutinins resemble those produced by bacteria more closely because both are thermolabile, decrease in titer on storage and are inhibited by normal serum. On the other hand, the virus hemagglutinins are more heat stable and are inhibited only by specific sera.

Occasionally the stool suspensions were bacteriologically sterile but generally *Escherichia coli*, *Staphylococcus*, yeasts or diphtheroids were isolated. Turbid saline suspensions of the various isolated organisms (containing approximately 1 billion per cc) were tested for agglutination with chick cells, group "O" human, rabbit and guinea pig cells. No hemagglutination was observed with any of the cells tested except the rabbit erythrocytes which agglutinated in relatively low titers (1:2 to 1:6 dilutions of the bacterial suspensions). These results are in accord with those recently

<sup>4</sup> Cox, H. R., et al., *J. Immunol.*, 1947, 56, 149.

TABLE I.  
Comparative Effects of Vitamin B<sub>12</sub> and Various Liver Fractions on the Growth of Immature Rats Fed Massive Doses of Desiccated Thyroid.

Supplements fed with basal ration	% thyroid	No. of animals	Initial body wt, g	Avg gain in body wt after 28 days of feeding,* g
None	.5	10	46.1	47.8 ± 4.0 (6)
Whole liver powder	.5	10	46.0	109.7 ± 2.1 (8)
Extracted liver residue	.5	10	46.0	103.4 ± 4.4 (9)
Liver concentrate 1-20	.5	10	46.1	63.3 ± 3.2 (9)
Vit. B <sub>12</sub>	.5	10	46.1	48.3 ± 3.5 (7)
None	.0	6	45.7	98.8 ± 5.8 (6)
Vit. B <sub>12</sub>	.0	6	45.8	97.6 ± 3.9 (6)

The values in parentheses indicate the number of animals that survived of which this is an average.

\* Including standard error of the mean calculated as follows:  $\sqrt{\frac{\sum d^2}{n}} / \sqrt{n}$  where "d" is the deviation from the mean and "n" is the number of observations.

contains one or more factors other than vitamin B<sub>12</sub> which were responsible for its anti-thyrototoxic effects under conditions of the present experiment.

Available data indicate that requirements for a number of nutrients are markedly increased in the hyperthyroid animal.<sup>13</sup> This is particularly true for some of the B vitamins. An increased requirement for thiamine,<sup>12,13</sup> pyridoxine,<sup>12,13</sup> pantothenic acid,<sup>12,13</sup> folic acid<sup>14</sup> and more recently vitamin B<sub>12</sub><sup>7,8</sup> has been demonstrated following administration of large doses of thyroactive substances. In addition to the above, requirements are increased for at least one additional factor in the hyperthyroid animal. This substance, which has been termed the "antithyrototoxic factor of liver", is retained in the water-insoluble fraction of whole liver powder. Recent findings<sup>2,3</sup> with those of the present experiment indicate that this factor is distinct from any of the known nutrients including vitamin B<sub>12</sub>.<sup>15</sup>

**Summary.** Growth was markedly reduced in hyperthyroid rats fed purified rations containing casein as the dietary protein and sucrose as the dietary carbohydrate. The re-

tardation in growth was completely counteracted by the administration of a water-insoluble fraction of liver. Crystalline vitamin B<sub>12</sub> was ineffective. The protective factor in liver is distinct from any of the known nutrients including vitamin B<sub>12</sub>.

While this paper was in press Bethell and Lardy<sup>15</sup> reported that crystalline vitamin B<sub>12</sub> partially counteracted the growth retardation of immature male rats fed massive doses of thyroid in a ration similar to that employed in the present experiment. Growth was significantly less, however, than that occurring in animals fed a similar diet containing 10% whole liver powder. It would appear that Bethell and Lardy were dealing with a multiple deficiency induced by thyroid feeding. Vitamin B<sub>12</sub> was one of the deficient factors, but optimum growth was not obtained unless animals were supplied an additional factor or factors present in whole liver. Emerson<sup>16</sup> has recently found that Vitamin B<sub>12</sub> may be stored in considerable amounts in the tissues of the young rat during the period of pregnancy and lactation. It is not unlikely that differences in the growth of immature hyperthyroid rats fed vitamin B<sub>12</sub> may be due, at least in part, to the vitamin B<sub>12</sub> content of the pre-test dietary regime.

<sup>15</sup> Bethell, J. J., and Lardy, H. A., *J. Nutrition*, 1949, **37**, 495.

<sup>16</sup> Emerson, G. A., *Fed. Proc.*, 1949, **8**, 381.

<sup>13</sup> Drill, V. A., *Physiol. Rev.*, 1943, **23**, 355.

<sup>14</sup> Martin, G. J., *Amer. J. Dig. Dis.*, 1947, **14**, 341.

B<sub>12</sub>.<sup>7,8</sup> On rations containing casein as the dietary protein and sucrose as the dietary carbohydrate, however, the retardation in growth following administration of massive doses of thyroactive substances appears to be due to a deficiency of some other factor. This latter substance which has been termed the "antithyrototoxic factor of liver" is present in considerable concentration in the water-insoluble fraction of liver.<sup>2,9</sup> It is not present in significant amounts in liver fractions with vitamin B<sub>12</sub> activity or other concentrates of vitamin B<sub>12</sub>.<sup>10</sup> In the present communication further data are presented concerning the ineffectiveness of crystalline vitamin B<sub>12</sub> as a source of antithyrototoxic factor for the immature hyperthyroid rat.

*Procedure and results.* The basal ration employed in the present experiment consisted of sucrose, 73.0%, casein,<sup>†</sup> 22.0%, salt mixture,<sup>‡</sup> 4.5%; and U.S.P. desiccated thyroid,<sup>§</sup> 0.5%. To each kg of the above were added the following synthetic vitamins: thiamine hydrochloride, 72 mg; riboflavin, 9 mg, pyridoxine hydrochloride, 15 mg, calcium pantothenate, 67.2 mg, nicotinic acid, 60 mg, 2-methyl-naphthoquinone, 5 mg and choline chloride 1.2 g.<sup>||</sup> Each rat also received 3 times weekly the following supplement: cot-

tonseed oil (Wesson) 500 mg, alpha-tocopherol acetate 1.5 mg and a vitamin A-D concentrate containing 50 U.S.P. units of vitamin A and 5 U.S.P. units of vitamin D.<sup>¶</sup> In addition to the basal ration the following diets were also employed, consisting of basal ration plus each of the following supplements: (1) 30  $\gamma$  of vitamin B<sub>12</sub> per kg of diet,<sup>\*\*</sup> (2) 10% whole liver powder,<sup>††</sup> (3) 10% extracted liver residue,<sup>‡‡</sup> and (4) 4% liver concentrate 1-20.<sup>§§</sup> The liver fractions were added in place of an equal amount of sucrose. In addition to the above, two control diets were also tested. These consisted of (1) basal ration with thyroid omitted and (2) a similar ration supplemented with 30  $\gamma$  of vitamin B<sub>12</sub> per kg of diet. Sucrose replaced thyroid in both of the latter rations. Sixty-two female rats of the Long-Evans strain were selected at 21 to 23 days of age and an average weight of 46.0 g for the present experiment. Animals were kept in metal cages with raised screen bottoms to prevent access to feces and were fed the above diets *ad lib*. Feeding was continued for 28 days or until death, whichever occurred sooner. Results are summarized in Table I.

In agreement with earlier findings whole liver powder completely counteracted the growth retardation of immature rats fed massive doses of thyroid. The protective factor was retained in the water-insoluble extracted liver residue. Little if any activity was present in the water-soluble liver concentrate 1-20. Crystalline vitamin B<sub>12</sub> was inactive. These findings indicate that extracted liver residue

<sup>7</sup> Nichol, C. A., Dietrich, L. S., Cravens, W. W., and Elvehjem, C. A., *Proc. Soc. Exp. Biol. and Med.*, 1949, **70**, 40.

<sup>8</sup> Emerson, G. A., *Proc. Soc. Exp. Biol. and Med.*, 1949, **70**, 392.

<sup>9</sup> Ershoff, B. H., and McWilliams, H. B., *Science*, 1948, **108**, 632.

<sup>10</sup> Ershoff, B. H., *Exp. Med. and Surg.*, 1948, **6**, 438.

<sup>†</sup> Vitamin Test Casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

<sup>‡</sup> Sure's Salt Mixture No. 1.<sup>11</sup>

<sup>11</sup> Sure, B. J., *J. Nutrition*, 1941, **22**, 499.

<sup>§</sup> Thyroid Powder, U.S.P., Armour and Co., Chicago, Ill.

<sup>||</sup> In view of the increased requirements for thiamine, pyridoxine and pantothenic acid in the hyperthyroid rat,<sup>12</sup> the B vitamins in the present experiment were administered in excessive amounts in order to assure an adequacy of these factors in the diet.

<sup>12</sup> Drill, V. A., and Overman, R., *Am. J. Physiol.*, 1942, **135**, 474.

<sup>¶</sup> Nopco Fish Oil Concentrate, assaying 800,000 U.S.P. units of vitamin A and 80,000 U.S.P. units of vitamin D per gram.

<sup>\*\*</sup> The vitamin B<sub>12</sub> employed in the present experiment was obtained from Merck and Co., Rahway, N. J.

<sup>††</sup> Whole Dried Liver Powder, Armour and Co., Chicago, Ill.

<sup>‡‡</sup> Extracted Liver Residue, Wilson Laboratories, Chicago, Ill. This material consists of the coagulated, water-insoluble material remaining after the removal of the extractable water-soluble substances.

<sup>§§</sup> Liver Concentrate Powder 1-20, Wilson Laboratories, Chicago, Ill. This material consists of the water-extractable material of raw liver.

TABLE I.  
Comparison of the Amount of Ascites Formed in a Group of Rats with Partial Ligation of the Portal Vein and in an Operated Control Group.

Operation	No. of rats	Rats with ascites			
		1-4 cc		>4 cc	
		No.	%	No.	%
Intestinal manipulation	26	0	0	0	0
Portal vein ligation	110	60	55	16	14

abdomen developed in only 3 of the rats. The 2-stage complete ligation of the portal vein did not cause a higher incidence of ascites.

In view of the fact that the occurrence of ascites appeared to be quite unpredictable in the rats with partial ligation of the portal vein, the pressure in the portal circuit was determined. This was carried out by means of a simple small diameter manometer that was connected to a needle which was inserted into branches of the mesenteric veins. Heparinized saline was used in the manometer and the results obtained were corrected for capillarity. Anaesthesia was found to increase considerably the pressure in the portal system and the pressure could be made to rise and fall according to the degree of anesthesia. It was possible, however, to bring all the animals to a constant stage of anaesthesia so that the pressures could be compared. Normal animals gave pressures ranging from 100-160 mm H<sub>2</sub>O. All animals with partial ligation showed values which were higher than the highest normal. Table II shows the portal pressure in 9 rats along with the amount of ascites. It is evident that there is no direct correlation. However, there was a general tendency for the animals with the higher pressures to have more ascites. The cause of the large amount

TABLE II.  
Comparison of the Portal Pressure and the Volume of Ascitic Fluid in 9 Rats Operated on 6 Days After Partial Ligation of the Portal Vein.

Rat No.	Portal pressure, mm H <sub>2</sub> O (Normal 100-160)	Ascites, cc
1	350	4
2	320	2
3	310	3
4	280	0
5	270	10
6	250	0
7	230	2
8	220	0
9	210	0

of ascites in some of the animals was not clear. It appeared as though other factors were operating in these animals in addition to increased pressure. With time the portal pressure gradually declined and usually fell to a level just above normal at the end of 3 weeks.

*Summary.* Partial ligation of the portal vein resulted in the development of a small amount of ascites in 69% of a group of 110 rats. All rats showed an increase in portal pressure but the degree of increase did not correlate well with the amount of ascites.

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# 17137. Increased Portal Pressure and Ascites in Rats Following Ligation of Portal Vein.

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Numerous reports have appeared in the literature<sup>1-3</sup> concerning the failure to produce ascites in animals by ligation of the portal vein. These results have caused some people to minimize the importance of increased portal pressure as a factor in the mechanism of ascites in patients with cirrhosis of the liver. Recently Blakemore<sup>4</sup> and Linton<sup>5</sup> have demonstrated that ascites sometimes disappears following anastomoses between the portal and the general venous circulation. Portal pressures 3 and 4 times the normal range have been found at operation in patients with cirrhosis accompanied by ascites. However, the exact role of elevated portal pressure in the production of ascites is not clear.

The present experiments were undertaken to assess the influence of hypoproteinemia, increased Na intake and antidiuretic substances on the production of ascites in rats with elevated portal pressure. It was found that partial ligation of the portal vein alone produced some ascites in a large percentage of the rats. These animals were demonstrated to have increased portal pressure. The additive effect of the other procedures on ascites will be described in another communication.

**Experimental Procedure and Results.** Adult rats weighing 200-300 g were operated on under ether anesthesia and the portal vein above the splenic vein was ligated to approximately  $\frac{1}{4}$  its original circumference. This was carried out by exteriorizing the portal vein and tying it off completely. Then a small probe was inserted in the loose knot and it

was dilated to the desired circumference. If the ligation was complete, all animals died within a few hours. The portal pressure in such animals reached values as high as 1,000 mm H<sub>2</sub>O and numerous small vessels were ruptured. In addition, there was marked engorgement of blood, giving a dark red color to the intestines. In the partial ligation an effort was made to allow just enough portal blood to pass to prevent the death of the animal. The color of the intestines helped to determine the degree of obstruction. It was possible to raise the portal pressure as much as 200 mm of water by the partial ligation and still obtain 85% survival. Complete ligation of the portal vein was carried out in 2 stages in 42 rats by the method of Whitaker.<sup>6</sup> This was a somewhat cumbersome procedure and did not give significantly higher portal pressures than the one-stage partial ligation. A 60% survival rate was obtained for the 2-stage complete ligation.

Following partial ligation of the portal vein, ascites was carefully sought both by laparotomies and by placing a needle in the abdomen. Ascites was found in 69% of the animals. Table I shows the incidence of ascites in 110 rats which received the partial ligation as compared to a control group which received only intestinal manipulation at the time of operation. The fluid obtained resembled human ascitic fluid with a slightly higher protein content (an average of 2.2%). The ascites was found to develop usually on the second day after the operation, reach a peak at approximately the 6th day and then gradually disappear so that few of the rats had any ascites after the 14th day. The ascites could easily be missed and was often not evident from the weight changes of the animals. Massive ascites requiring removal of fluid from the

<sup>1</sup> Eck, N. V., *Foyena Med. J.*, 1877, 130, 1.

<sup>2</sup> Bollman, J. L., *Trans. Liver Injury Conference*, Josiah Macy, Jr. Foundation, 1948, 7, 21.

<sup>3</sup> Milnes, R. F., and Child, C. G., *Proc. Soc. Exp. Biol. and Med.*, 1949, 70, 332.

<sup>4</sup> Blakemore, A. H., *New York State J. Med.*, 1947, 47, 479.

<sup>5</sup> Linton, R. R., Hardy, I. B., and Volwiler, W., *Surg., Gynec. and Obst.*, 1948, 87, 129.

<sup>6</sup> Whitaker, W. L., *Proc. Soc. Exp. Biol. and Med.*, 1946, 61, 420.

TABLE I.  
X-ray Dosage-Mortality in Rats.

r.	No. rats	50% dead (days)	% mortality (30 days)
1000	8	6	100
800	32	8	90
750	8	8	100
715	8	8	100
670	8	16	53
655	235	15	79
640	83	30	41
625	94	45	62
610	8		0
500	17		6
	501		

convenient endpoint for expressing the data, although in most instances observations were continued for 45-60 days or longer. For all practical purposes the present mortality data include indefinite survival.

Table I lists the results on 501 rats.

The dosages (column 1) were plotted against per cent mortality (column 4) as shown in Fig. 1.

Fig. 2 relates the survival time to per cent

mortality, for each dosage. Because of overlapping, it was found most useful to pool the data as indicated.

The time in days lapsing before 50% of the animals died was plotted against dosage, in Fig. 3. This relationship has been found useful in assessing the effects of drugs on radiation disease.<sup>1</sup>

When the data were plotted by Miller and Tainter's modification<sup>7</sup> of Litchfield and Fertig's method,<sup>8</sup> on logarithmic-probit graph paper, the  $LD_{50}$  was approximately  $640 \pm 5$  r. However, it was difficult to fit the points to a satisfactory straight line by this method because of the variations in the steep part of the curve, and from Fig. 1 it is evident that from 610 to 655 r ( $\pm 3\%$ ), a 0 - 86% mortality may occur. The  $\pm 3\%$  is the calibrated limit of variation of the X-ray machine, so that any results from 0 to nearly 100% mortality could be expected within the " $LD_{50}$ " range because of the steepness of the slope in this range. Only unless very large numbers of animals were used together with radiation dose variation in the hundredth per cent range, could this portion of the curve be duplicated from one experiment to the next.

Table II lists some calculations from our data on the rat, compared with those of Ellinger<sup>9</sup> for goldfish, guinea pigs and mice, and of Tullis, *et al.*<sup>10</sup> for swine; of the rate

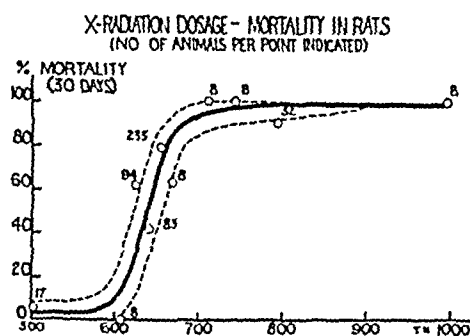


FIG. 1.

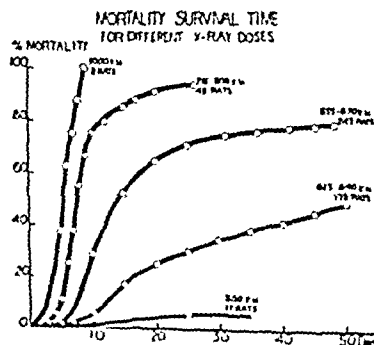


FIG. 2.

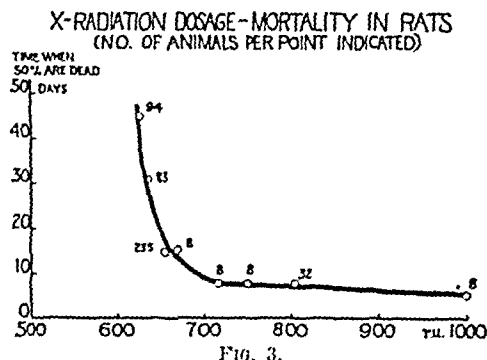


FIG. 3.

<sup>7</sup> Miller, L. C., and Tainter, M. L., *Proc. Soc. Exp. Biol. and Med.*, 1944, 57, 261.

<sup>8</sup> Litchfield, J. T., Jr., and Fertig, J. W., *Bull. Johns Hopkins Hosp.*, 1941, 60, 276.

<sup>9</sup> Ellinger, F., *Radiology*, 1940, 35, 563.

<sup>10</sup> Tullis, J. L., Tessmer, C. F., Cronkite, E. P., and Chambers, F. W., *Radiology*, 1949, 52, 396.

# 17138. Dosage-Mortality in Rats Given Total Body Roentgen Irradiation.\*

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In a previous communication<sup>1</sup> we indicated that certain flavonoid compounds may decrease the mortality of guinea pigs given approximately an LD<sub>50</sub> of total body roentgen radiation.

In attempts to extend these and other treatments to other species of small laboratory animals, some results have been obtained on dosage-mortality in the rat, which may be of value to others working on the biological effects of ionizing radiation.

We have been unable to find previous published mortality data for the rat other than a note by Potter,<sup>2</sup> who reported 100% death in 14 rats with total body radiation of 800 r. Prosser, *et al.*<sup>3</sup> state that the 30-day M.L.D. for single doses of x-radiation is 600 r† for rats but no particulars were given.

In our studies on the rat, over 500 animals have been used. The purpose of this communication is to report the dosage-mortality data for normal rats.

The rats used were of the Slonaker albino stock, from which the Wistar albino stock originated and which have been inbred in this laboratory since 1929.<sup>4</sup> Their food consisted of Purina laboratory chow available on the meshscreen cage floors and tap water from

bottles with glass spouts. In all cases the animals were healthy young males, average body weight 100-140 g.‡ Six animals were caged together. The room temperature was 78° ± 2°F.

The animals to be irradiated were placed in a ventilated 6-partitioned plywood box, 6 at a time. The dimensions of each compartment were 2" wide, 5" long (adjustable) and 3" deep. The partitions were 1/8" plywood and the top was 1/4" plywood perforated with holes. The bottoms were 1/4" hardware screen and beneath this was a layer of rice 3 inches in depth which was used to provide additional backscatter.§

The X-ray factors were: GE Model KX-3, 220-kv deep therapy unit, 200 kv, 20 MA, 1/2 mm Cu and 1 mm Al added filtration (HVL, 1.05 mm Cu) 52.5 cm target distance to top of box, 30.4 r/min. in air single exposure total body irradiation. The times were varied to give the desired dosage. The unit is calibrated semi-annually by a registered X-ray physicist. The variation in output over the past year has been a little under 3%. This of course does not ensure accurate control from day to day, but since control animals always were under the beam at the same time as the experimentals, this objection is not so serious.

It was found that if rats survived longer than 30 days they generally survived indefinitely. Hence, 30 days were taken as a

\* This investigation was supported by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service.

1 Clark, W. G., Uncapher, R. P., and Jordan, M. L., *Science*, 1948, **108**, 269.

2 Potter, J. C., *Radiology*, 1941, **37**, 724.

3 Prosser, C. L., Painter, E. S., Lisco, H., Brues, A., Jacobson, L. O., and Swift, M. N., *Radiology*, 1947, **49**, 299.

† A. H. Dowdy (personal communication) informed us that the Rochester Unit of the Manhattan District found an LD<sub>50</sub> of 650 r for Wistar rats, which closely agrees with the results reported below.

‡ MacKay, L. L., and MacKay, E. M., *Am. J. Physiol.*, 1927-8, **83**, 179.

§ A wider weight distribution should not be used, since Naiman<sup>5</sup> has shown a considerable effect of age on susceptibility of rats to total body x-radiation.

5 Naiman, D. N., *Am. J. Roentgenol.*, 1949, **61**, 95.

§ Ellinger<sup>6</sup> has shown in mice that additional backscatter of this nature reduces the steepness of the slope of the sigmoid mortality curve in the LD<sub>50</sub> range.

6 Ellinger, F., *Radiology*, 1945, **44**, 125.

observed that the serological reactions to a strain of virus of swine origin were similar to those obtained with strains isolated during the 1947 outbreak in the civilian population of the United States.<sup>2-4</sup> The evidence strongly suggests that this swine strain (Oti) is closely related to strains of human origin more recently studied which are being called A-prime.

*Materials and methods. Description of Virus Strains.* The virus preparations used in the agglutination inhibition tests were taken from infected allantoic fluid. The passage history of each strain is as follows: Type A RP8<sup>5</sup>—7 ferret, 593 mouse and 69 egg passages; A-prime Rhodes<sup>2</sup>—4 ferret, 7 mouse, and 13 egg passages; A-prime FM1<sup>3</sup> (obtained from Dr. J. E. Smadel)—37 mouse and 15 egg passages; Swine 1976 (obtained from Dr. R. E. Shope)—38 mouse and 19 egg passages; Swine Oti, a strain sent to Dr. R. E. Shope by Dr. M. Tsurumi of the Imperial University of Nagoya, Japan. He, in turn, had received it from Dr. Oti, who originally isolated it from swine in Korea in 1939. Dr. Shope noted that this strain was different in its behavior from the American swine strains, and after being received in this laboratory in 1944 after 179 mouse passages it was soon realized that it acted a great deal like a type A human strain. Since then it has been passed 33 times in eggs.

*Sera from Children.* There were 175 children in the institution where the vaccination study was carried out; 56 recognized cases of influenza appeared among them between late February, 1947 and the middle of April, 1947. Serum had been obtained from most of the children at the termination of the vaccination study, in early November 1946, four months before the outbreak of influenza. Be-

ginning one week after the last clinically recognized case, approximately seven months after the last previous sample, another specimen of serum was obtained from 52 children, eleven of whom had been noticeably ill during the epidemic period.

*Sera from Adults.* A group of 93 pairs of acute and convalescent sera were obtained from patients admitted to the student Health Service Infirmary of the University of Michigan during the period of the outbreak.<sup>2</sup> Fifty-eight of them had been inoculated with a vaccine of combined A and B influenza viruses.

*Sera from Animals.* All animal sera, with one exception, were from ferrets which received intranasal inoculations of the allantoic fluids containing the specific viruses. Sera were generally obtained before and 2 weeks after inoculation. Strict isolation technic was used and only single strains of virus were introduced into the ferret isolation unit to eliminate the possibility of cross-infection of the animals. One anti-serum prepared in rabbits (inoculated with the Oti strain in 1944) was used to check the identity of the current Oti line that was used in the tests. The results indicated that the current Oti line reacted the same as it had before any A-prime strains had been isolated in this laboratory.

*Serological Tests.* Antibody was measured by inhibition of agglutination of chicken erythrocytes. Four agglutinating units of virus were mixed with the serially diluted sera. A pattern method<sup>6</sup> of interpreting the endpoint was used. The titers are expressed as reciprocals of the modified means.<sup>7</sup>

Neutralizing antibody was measured by inoculating mice with serially diluted serum plus 500-1,000 MLD of the virus strains. The 50 per cent endpoints<sup>8</sup> were calculated on the basis of the lethal effect of virus during a ten-day observation period. Titers are recorded as final dilutions of serum.

*Results.* An extensive examination was made of the sera obtained from the children

<sup>2</sup> Francis, Thomas, Jr., Salk, Jonas E., and Quilligan, J. J., Jr., *Am. J. Pub. Health*, 1947, **37**, 1013.

<sup>3</sup> Sigel, M. M., Shaffer, F. W., Kirber, M. W., Light, A. B., and Henle, W., *J.A.M.A.*, 1948, **136**, 437.

<sup>4</sup> Smadel, J. E., *Bull. U. S. Army Med. Dept.*, 1947, **7**, 795.

<sup>5</sup> Francis, T., Jr., *Science*, 1934, **80**, 457.

<sup>6</sup> Salk, J. E., *J. Immunol.*, 1944, **49**, 87.

<sup>7</sup> Quilligan, J. J., Jr., and Francis, Thomas, Jr., *J. Clin. Invest.*, 1947, **26**, 1079.

<sup>8</sup> Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27**, 493.



TABLE II.  
Mortality Rate per r. Unit—Species Differences.

Species	No. animals. used	$dx/dy^*$	Reference
Rats	500	1.2%	Clark and Uncapher.
Guinea pigs	50	0.7	Ellinger <sup>6</sup>
Swine	32	0.5	Tullis <i>et al.</i> <sup>10</sup>
Mice	119	0.3	Ellinger <sup>6</sup>
Goldfish	774	0.08	Ellinger <sup>9</sup>

\*  $x = \% \text{ mortality}$  and  $y = \text{dosage in } r, \text{ in the } LD_{50} \text{ range.}$

of mortality per roentgen unit dose, in the steepest part of the mortality curves. This is an expression of the slope,  $dx/dy$ , where  $x$  represents unit per cent mortality and  $y$  the unit dose.

This means that 1.2% of the rats died per unit increment in dosage of X-rays, where the other species died at a lower rate per dosage increment.

It will be interesting to compare these data with those for the dog, rabbit, monkey, cat and other species.

It occurs to us that in studies directed toward the therapy or protection by pre-treatment of animals given total body ionizing radiation, that if the mortality curves are quite steep, as in the rat, dosages in the " $LD_{50}$ " range may not be permissible, and that dosages should be given which consistently kill 100% of the untreated animals, so that an estimation of protection could be made by both the time required to kill 50% of each group, as shown in Fig. 3, and by the

per cent final mortality. If the slope is not so steep ( $dx/dy$  is small) and if sufficient animals are used, it would be advisable to include at least 3 dosages,  $LD_{100}$ ,  $LD_{50+10}$  and  $LD_{20+30}$ .

**Summary.** The  $LD_{50}$  for total body X-radiation in Wistar rats is  $640 \pm 5 r$  with a 30-day endpoint. The time elapsing before 50% mortality occurs at different doses is a more reliable measure of mortality than the dosage-mortality curve or the dosage-survival time curves for different X-ray doses. The dosage-mortality curve of rats has such a steep slope in the  $LD_{50}$  range that variations in mortality of from 0 to nearly 100% may occur in this range because of variations in animals and in the X-ray machine output. Therefore in studies directed toward therapy or protection of animals given total body ionizing radiation, if the mortality curves are extremely steep as in the rat, dosages in the  $LD_{50}$  range should not be used. Other measures which might be employed are discussed.

We wish to express our gratitude to Dr. F. Ellinger, Director of Radiological Research, Naval Medical Research Institute; to Dr. A. H. Dowdy, chairman, Department of Radiology, School of Medicine, University of Calif.; and to Dr. V. P. Bond and Peggy Smith of the Navy Radiological Defense Laboratory, San Francisco Naval Shipyard, for reading this manuscript and making certain suggestions.

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### 17139. Resemblance of a Strain of Swine Influenza Virus to Human A-prime Strains.\*

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In the course of studies in a group of children vaccinated with the Type A PR8

strain of influenza virus, an outbreak of influenza occurred in that population.<sup>1</sup> The children's sera were being tested against different strains of influenza virus, and it was

\* This investigation was conducted under the auspices of the Commission on Influenza, Army Epidemiology Board, Office of the Surgeon General, U. S. Army, Washington, D.C.

<sup>1</sup> Quilligan, J. J., Jr., Minuse, Elva, and Francis, Thomas, Jr., *J. Clin. Invest.*, 1949, 27, 572.

TABLE IV.  
Cross Hemagglutination-Inhibition and Mouse Neutralization Tests with Allantoic Fluid Antigens and Specific Ferret Antisera.

Ferret sera	Serum stage	Virus strains									
		PR8		Rhodes		FM1		Oti		Swine 1976	
		F7M593E69		F4M7E23		M37E15		M179E33		M38E19	
		HI	MN	HI	MN	HI	MN	HI	MN	HI	MN
G28	N	<32	<4	<32	—	—	—	<32	—	<32	—
PR8-F105	I	4096	1024	<32	24	—	—	<32	<4	<32	<4
G12	N	<32	—	32	—	<32	—	<32	—	<32	—
PR8-F105	I	1024	—	32	—	<32	—	<32	—	<32	—
K24	N	<32	—	<32	<4	—	—	<32	—	<32	—
Rho-F4M7E12	I	<32	4	2048	512	—	—	<32	<4	<32	<4
K25	N	<32	—	<32	—	<32	—	<32	—	<32	—
Rho-F4M7E12	I	<32	—	2048	—	128	—	<32	—	<32	—
K46	N	<32	—	<32	—	<32	—	<32	—	<32	—
FM1-M37E14	I	<32	—	2048	—	1024	—	<32	—	<32	—
K31	N	<32	—	<32	—	—	—	<32	<4	<32	—
Oti-M179E33	I	128	128	128	128	—	—	2048	512	32	<4
K30	N	<32	—	<32	—	<32	—	<32	—	<32	—
Oti-M179E33	I	128	—	128	—	<32	—	2048	—	<32	—
K37	N	<32	—	—	—	—	—	<32	—	<32	<4
Swine 1976-M52	I	<32	<4	—	4	—	—	<32	<4	512	1280
K38	N	<32	—	32	—	32	—	<32	—	<32	—
Swine 1976-M52	I	<32	—	64	—	32	—	<32	—	256	—

F = Ferret. M = Mouse. E = Chick embryo.

Numbers after letters correspond to number of passages in animals.

N = Normal serum. I = Immune serum.

All titers are reciprocals of one.

— = Not done. HI = Hemagglutination-inhibition titer. MN = Neutralization titer in mice.

In this respect the response of the children appeared to be clearly more specific.

A further comparison of the response of the children and the adults to the A-prime infection is listed in Table III as the per cent showing incremental change in titer. This demonstrated again greater specificity on the part of the children with a rather striking parallel in the pattern of response to the Rhodes and Oti strains. The results in the adult group were less pronounced but followed the same pattern. As previously noted vaccination tends to mask the effect of A-prime infection on the antibody responses to the PR8 strain.

It appears that the Oti strain, when tested against human sera from individuals who had experienced A-prime infection, showed antibody reactions which strongly suggest that it is an A-prime strain.

*Results of Studies Using Specific Animal Antisera.* The results in the two illness groups indicated the desirability of further investigation of the immunological characteristics of the Oti strain. Table IV shows the relation of cross-inhibition tests using specific ferret antisera against Type A, A-prime and swine strains. It is readily apparent that the Oti strain is more closely related to the A-prime and A strains than to the Swine 1976 strain. Furthermore, neutralization tests in mice paralleled the agglutination-inhibition tests. Animals inoculated with the Oti strain developed high antibody titers against the Oti strain and slightly less antibody against the Type A or A-prime strains while exhibiting no antibody against the swine strain. Animals inoculated with the A and A-prime strains had no antibody against either the Oti or the swine strain 1976. These results

TABLE I.  
Effect of A-prime Influenza Virus Infection on Serological Response of the Children Expressed as the Modified Mean Antibody Titers.

Sera from	Stage	No.	Modified mean titers			
			PR8	Rho	Oti	Swine 1976
Children	Pre-infection	52	716	43	38	24
	Post-infection	52	625	160	136	35

TABLE II.  
Comparison of Antibody Titers of Vaccinated and Unvaccinated Members of the University Group.

	Virus strains							
	PR8		Rhodes		Oti		Swine 1976	
	Vac.	Unvac.	Vac.	Unvac.	Vac.	Unvac.	Vac.	Unvac.
No. of sera tested	57	35	50	35	58	35	57	35
Acute*	371	77	40	34	141	63	50	35
Conv.*	722	230	114	106	415	242	106	90
Fold-inc.	1.9×	3×	2.8×	3×	2.9×	3.8×	2.1×	2.6×
in means								

\* Modified mean titer.

TABLE III.  
Effect of A-prime Influenza Virus Infection on Serological Response of Two Groups of Individuals Expressed as the % Showing Incremental Changes in Titer.

Group	Virus strain	Decrease or no change	2-fold rise	4-fold or greater rise	Total No.
University students	PR8	30	33	37	92
	Rho	21	29	50	93
	Oti	22	26	52	93
	Swine 1976	29	41	30	91
Children	PR8	73	11	16	37
	Rho	18	22	61	51
	Oti	22	15	63	51
	Swine 1976	53	29	18	51

following repeated inoculations of the univalent vaccine.<sup>1</sup> It was found that but a minimal rise in antibody to the 1976 strain of swine virus occurred and the response as measured with the Oti strain was small. However, when the sera taken after the outbreak of influenza were compared with those obtained earlier a sharp increase in titer to the Oti strain was noted (Table I). There was no significant change in the low titer against the Swine 1976 strain, nor in the high post-vaccination titer against the PR8 strain. The results with the Oti strain were essentially parallel to those measured by the Rhodes strain (A-prime) isolated during the 1947 epidemic. Since all the children in the

group were vaccinated with PR8 the lack of further response to that strain could be readily ascribed to the vaccination.

The group of University students who had been sick comprised both vaccinated and unvaccinated persons. The highest convalescent mean titer and the largest incremental increase in mean titer among the unvaccinated were observed against the Oti strain but threefold rises also occurred against both the PR8 and the 1947 Rhodes strain (Table II). A definite rise to Swine 1976 strain also took place. In the vaccinated group, there was also an increase in titer against all strains used, although the incremental rises were greatest against the Oti and Rhodes strains.



differed from the findings in man where experience with an A-prime infection caused rises in anti-Oti antibody. Using the more specific animal sera less evidence of cross inhibition between the Oti and other strains was obtained than between the A-prime strains of Rhodes and FMI. The Oti strain did not crossreact with the antisera to the PR8, Type A, strain. It should be pointed out, however, that anti-Oti serum gave as much inhibition of the Rhodes strain as anti-Rhodes serum did with the FMI strain. Further, there is considerable variation in the amount of cross reaction obtainable with strains of the A-prime group. In any event, it appears that this strain behaves like a type A strain and certainly does not have the characteristics of the swine strains described by Shope.<sup>10</sup> These data, while not decisive, taken together with the results obtained with human sera seem to warrant the inclusion of the Oti strain with the A-prime groups of 1947.

**Discussion.** The history of the Oti strain seems sufficiently accurate to accept the fact that it was originally isolated from infected swine in Korea where swine influenza is recognized largely as a low grade endemic disease and differs from the sharp epidemics described in the United States. The data presented in this paper call attention to the definite antigenic resemblance of this swine strain, isolated in 1939, to strains such as the A-prime group first prominently encountered in human disease in 1947. It may be that some of the peculiarities of that group of strains are related to their earlier adaptation in the animal host. If subsequent studies should increase

the possibility that these infections of man arose from a swine host, it would represent a reversal of the sequence of host infection from that suggested earlier, by Koen<sup>9</sup> and Shope<sup>10</sup> for swine influenza recognized in the United States. On the other hand, the virus may have been of human origin, fortuitously detected in swine.

**Conclusions.** 1. A strain of swine influenza virus (Oti), isolated in 1939, has been re-investigated by serological means and found antigenically to resemble some members of the human A-prime influenza virus group.

2. Significant increases in the levels of antibody against this swine strain of influenza virus were detected in the convalescent blood specimens of children who had had experience during the A-prime influenza epidemic of early 1947. High titers of antibody were also found against the current A-prime strains.

3. Adults involved in the same epidemic also showed significant antibody increases against the same swine strain and the current A-prime and Type A strains.

4. Results of cross hemagglutination inhibition tests with specific ferret anti-sera supported the observations made from the serological tests with human sera.

5. The relationship of this swine strain to members of the A-prime group has been discussed.

<sup>9</sup> Dorset, M., McBryde, C. N., and Niles, W. B., *J. Am. Vet. Assn.*, 1922-23, 62, 162.

<sup>10</sup> Shope, R. E., *Harvey Lectures*, 1935-36, 31, 183.

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## 17140. The Hyperglycemic Action of Some Analogs of Epinephrine

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The preparation in the chemical laboratories of this Institute of a series of compounds

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to be studied for bronchodilator action has made available to us a group of epinephrine analogs, all of which have this action to a desirable degree. They also lack some of the

TABLE I.  
 Hyperglycemic Action of Some Epinephrine Analogs in the Rabbit.

Compound name or No.	Dose,* mg/kg	No. of rabbits	Rise in blood sugar, mg% Avg	Range	Time of peak rise, hr after inj.
l-epinephrine	0.15	3	106	18-157	3
"	0.3	3	207	200-212	3
"	0.5	3	495	462-552	2,4§
Win 162	1	3	11	7-14	2†
"	2	3	20	14-31	2
"	5	3	50	41-65	2
"	10	6	76	56-96	2
l-arterenol	0.25	2	40	23-57	1
"	0.5	4	35	18-44	2§
"	1	3	96	53-149	3
"	2	6	176	52-230	2
d-arterenol	2	4	17	5-31	2
"	10	8	47	0-86	2-3‡
Win 515	10	4	61	43-106	1-2‡
"	20	6	83	25-145	1-3‡
Isuprel	10	10	18	0-37	1-4‡§
"	25	6	28	0-45	1§
Win 3046	5	5	52	20-85	1
"	10	5	84	40-140	1-2‡
"	20	6	71	36-98	1
Controls	0	8	2	-2-10	—

\* All doses were calculated in terms of the free base.

† After it was established preliminarily that the peak response was at 2 hours after injection the subsequent observations were made only at that time.

‡ In these cases about as many animals showed peak responses at one time as another.

§ No observation was made at 3 hours in these cases.

this average peak are recorded in the Table.

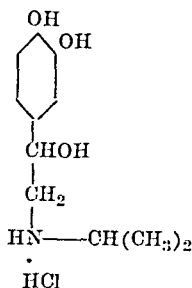
**Conclusions.** Of the several compounds tested, only l-arterenol has a hyperglycemic action of the same order as that of l-epinephrine. Its activity can be placed at about  $\frac{1}{8}$  of that of epinephrine, while the d-isomer has about  $\frac{1}{20}$  of the activity of the l-isomer. Sahyun<sup>14</sup> found that dl-arterenol, 1 mg/kg, produced an average rise in blood sugar of 78 mg% 3 hrs after subcutaneous injection in 7 rabbits. This is considerably more activity than would be calculated from our observa-

tions on the separate isomers. However, if one of his rabbits (No. 8, Table II) is eliminated from consideration, the average rise becomes only 61 mg%, and the discrepancy is much less. Of the other compounds studied, Isuprel is clearly the least active. The approximate hyperglycemic activity of the whole series of compounds, taking l-epinephrine as 100, is as follows (all figures given have been calculated in terms of the free base): l-arterenol, 12; Win 3046, 1.7; Butanefrine, 1.6; Win 515, 0.7; d-arterenol, 0.6; and Isuprel, 0.12.

<sup>14</sup> Sahyun, M., *Arch. Intern. Pharm.*, 1933, 45, 285.

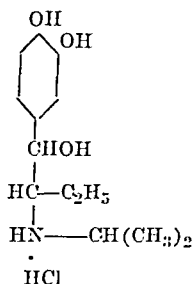
Win 516-2

Isuprel<sup>†</sup> hydrochloride  
[dl-1-(3,4-dihydroxy-  
phenyl)-2-isopropylamino-  
ethanol-hydrochloride]

Scheuing and Thom<sup>11</sup>

Win 3046

dl-1-(3,4-dihydroxy-  
phenyl)-2-isopropylamino-  
1-butanol hydrochloride

Bockmühl *et al.*<sup>10</sup>

\* Laboratory system of classification.

† Registered trade-marked names of Winthrop-Stearns Inc.

**Experimental methods.** The compounds to be tested were injected subcutaneously into healthy adult rabbits (wt. 1.5-4 kg), following an 18 hr fast. They were administered in a volume of 1 cc of 0.9% NaCl/kg except in cases where solubility limitations required a larger volume. Control animals received the

saline injection only. Blood samples were taken from the ear vein and were analyzed for glucose by the Folin-Malmros method,<sup>12</sup> or by a slight modification<sup>†</sup> of the Schales and Schales method.<sup>13</sup> Samples were usually taken at 1, 2, 3, and 5 hours after the injection. Analyses were also made before the injection, and any animal which gave a fasting blood sugar level of 130 mg% or more was rejected from that test. The results are presented in Table I. For purposes of simplification of the Table, only the peak rise of blood sugar, the extreme range of the rises in the several animals, and the approximate time of

<sup>1</sup> Lands, A. M., Nash, V. L., Dertinger, B. L., Granger, H. R., and McCarthy, H. M., *J. Pharm. and Exp. Therap.*, 1948, **92**, 369.

<sup>2</sup> Tainter, M. L., Cameron, W. M., Whitsell, L. J., and Hartman, M. M., *ibid.*, 1944, **31**, 269.

<sup>3</sup> Lands, A. M., Siegmund, O., and Ananenko, E., *Fed. Proc.*, 1949, **8**, 312.

<sup>4</sup> Tullar, B. F., *J.A.C.S.*, 1948, **70**, 2067.

<sup>5</sup> Ludueña, F. P., Ananenko, E., Siegmund, O., and Miller, L. C., *J. Pharm. and Exp. Therap.*, 1949, **95**, 155.

<sup>6</sup> Gaddum, J. H., and Goodwin, L. G., *J. Physiol.*, 1947, **105**, 357.

<sup>7</sup> West, G. B., *Pharm. J.*, 1947, **158**, 6.

<sup>8</sup> von Euler, U. S., *Science*, 1948, **107**, 422.

<sup>9</sup> Suter, C. M., and Ruddy, A. W., *J. A. C. S.*, 1944, **66**, 747.

<sup>10</sup> Bockmühl, M., Ehrhart, G., and Stein, L., U. S. Patent No. 2,083,001.

<sup>11</sup> Scheuing, G., and Thomä, O., U. S. Patent No. 2,308,232.

<sup>12</sup> Folin, O., and Malmros, H., *J. Biol. Chem.*, 1929, **83**, 115; Horvath, S. M., and Knehr, C. A., *ibid.*, 1941, **140**, 869.

<sup>†</sup> The authors state simply that a Folin-Wu filtrate is to be used. The filtrate was therefore prepared as follows: to 0.2 cc blood add 3.8 cc water followed by 3 cc of 0.67% sodium tungstate and 3 cc of 0.045 N. sulfuric acid. Centrifuge, and take 5 cc for analysis. If the blood sugar is found to exceed 300 mg %, the analysis is repeated on 2 cc of filtrate.

<sup>13</sup> Schales, O., and Schales, S. S., *Arch. Biochem.*, 1945, **8**, 285.

# 17142. Effect of Cortical Extract and Other Agents on Eosinophilia of Mice Infected with *Trichina spiralis*.\*

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An absolute reduction in the number of circulating eosinophiles occurs within a few hours following the stimulation of the human adrenal cortex with adrenotrophic hormone.<sup>1</sup> The same effect can be induced by the administration of cortical hormone, or epinephrine<sup>2</sup> which presumably stimulates anterior pituitary secretion of adrenotrophic hormone. Lymphopenia accompanies the drop in eosinophiles.<sup>3</sup>

A test animal in which eosinopenia can be readily determined for bioassay of adrenotrophic activity is desirable. In the mouse the normal level of blood eosinophiles is so low that normal animals cannot be utilized to good advantage. However, within 3-4 weeks after forcible ingestion of trichina-infected meat the mouse exhibits a decided eosinophilia which persists at a rather constant level for a matter of weeks. It was the purpose of this investigation to determine whether such infected animals might serve as a test object for hematologic assay of adrenocortical activity and/or function. The procedure was the following:

1. Young adult male mice were force-fed pectoral muscle from trichinous hosts (infected rats);<sup>‡</sup> eosinophilia of 4-20% was present

\* This investigation has been aided by grants from the Donner Foundation and the Graduate School of the University of Minnesota.

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<sup>1</sup> Thorn, G. W., Forsham, P. H., Recant, L., and Hills, A. G., Program Assn. Study Internal Secretions, 1948, 19.

<sup>2</sup> Recant, L., Forsham, P. H., and Thorn, G. W., Program Assn. Study Internal Secretions, 1948, 19.

<sup>3</sup> Hills, A. G., Forsham, P. H., Finch, C. A., Blood, 1948, 3, 755.

‡ The author is indebted to Dr. Franklin Wallace of the Department of Zoology, University of Minnesota, for supplying rats infected with trichina.

within 4 weeks and persisted for at least 5 weeks.

2. Total and differential counts, the latter on at least 100 cells, stained with Giemsa or Wright's stain, were made on the tail blood of unanesthetized animals.

3. The test material was then administered and counts were made after 6 and 24 or 48 hours.

The materials tested for their effect on the trichina-induced eosinophilia were whole adrenal cortex in oil, testosterone propionate, estradiol dipropionate, epinephrine, urethane, and peanut oil.<sup>§</sup> Crystalline hormones were dissolved in peanut oil, urethane in water.

Within 6 hours after the subcutaneous administration of cortical extract (0.1 cc lipo-adrenal cortex—Upjohn) the percentage of circulating eosinophiles had dropped from 12.7 to 1.6 (average of 7 animals). In this series of animals the decline in total white blood cell count averaged 5700 cells/mm<sup>3</sup>, in eosinophiles 1800, and in lymphocytes 2900. The initial counts averaged 16,400. Preliminary findings on 4 intact and 2 adrenalectomized trichinous mice injected subcutaneously with sex steroids (70 µg testosterone propionate and 25 µg estradiol dipropionate) revealed that within 6 hours following injection, except in one animal, the percentages of the white blood cells were not significantly affected. This was true also for the one adrenalectomized and 4 intact trichinous mice which received injections of peanut oil.

The absolute number and the percentage of circulating eosinophiles were reduced in trichinous mice by the subcutaneous administration of epinephrine (0.02 mg/100 g hourly for 4 hours). The drop in eosinophiles (at

§ The author is indebted to Dr. F. E. Houghton of Ciba Pharmaceutical Products, through whose courtesy the sex hormones were made available, and to Dr. L. E. Josselyn of Abbott Laboratories for supplying urethane.



17141. Further Investigations on *Donovania granulomatis*.

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It has been suggested elsewhere<sup>1,2</sup> that *Donovania granulomatis*, the etiological agent of granuloma inguinale,<sup>3</sup> may be a member of the tribe *Eschericheae* and be related to the genus *Klebsiella*. In this connection it was of interest to examine the carbohydrate fermentation reactions of *D. granulomatis*. Members of the *Klebsiella* group are known to vary widely in their capacity to ferment sugars, the *K. pneumoniae* group being the most active and *K. rhinoscleromatis* the least.

For the present investigation both solid and fluid media were used. These have been described in detail elsewhere.<sup>4</sup> In brief, the fluid medium was a beef heart infusion broth without the usual added glucose but with the various sugars listed in Table I added to a final concentration of 1% and with Andrade's indicator. The solid medium was a Levinthal beef heart agar again without the usual added glucose but with the same sugars to a final concentration of 1% and with Andrade's indicator. The results are shown in Table I.

All fermentation tests were held for 4 weeks. It will be noted that in broth only the 3 monosaccharides, glucose, fructose and galactose were fermented, while on solid medium there was also a delayed fermentation of maltose. In no case was any gas produced. In general *D. granulomatis* would seem to be even less active than *K. rhinoscleromatis* but from the point of view of these biochemical reactions could fit into the pattern of the *Klebsiellae*.

TABLE I.  
Fermentation Reactions of *D. granulomatis*.

	Broth	Agar
Glucose	+	+
Fructose	+	+
Galactose	+	+
Maltose	0	+
Sucrose	0	0
Lactose	0	0
Mannitol	0	0
Inositol	0	0

\* Indicates that signs of acid formation were first noted on the 5th day.

Further studies have been carried out on the sensitivity of *D. granulomatis* to the different streptomycins. The *in vitro* sensitivity has proved to be particularly high, over 3 times as high as the next most sensitive organism. In Table II the minimum inhibiting concentrations of the pure streptomycins are shown and compared to the next most sensitive organism encountered—the BCG strain of *M. tuberculosis* var. *bovis*—and to *K. pneumoniae*, an organism of greater than average sensitivity.

*Summary.* *D. granulomatis* shows delayed fermentation of a few carbohydrates, chiefly monosaccharides, without gas formation. In this respect it resembles *K. rhinoscleromatis*. Its sensitivity is high against all the pure streptomycins and cases of granuloma inguinale should react as favorably to treatment with dihydrostreptomycin as to treatment with streptomycin itself.

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TABLE II.  
*In vitro* Sensitivity Against Pure Streptomycins.

Organism	Streptomycin	Dihydrostreptomycin	Mannosidostreptomycin	Dihydromannosidostreptomycin
<i>D. granulomatis</i>	0.167	0.151	0.595	0.446
<i>K. pneumoniae</i>	1.76	1.76	6.39	6.59
<i>M. tuberculosis</i> (strain BCG)	0.52	0.55	1.9	1.7

<sup>1</sup> Rake, G., *Am. J. Syph., Gen. and Ven. Dis.*, 1948, **32**, 150.

<sup>2</sup> Rake, G., *J. Bact.*, 1948, **55**, 865.

<sup>3</sup> Anderson, K., *Science*, 1943, **97**, 560.

<sup>4</sup> Rake, G., and Oskay, J. J., *J. Bact.*, 1948, **55**, 667.

TABLE I.  
Comparative Effects of Pteroylglutamic Acid, Liver Extract and Vitamin B<sub>12</sub> on the Growth of Rous Sarcoma.

Supplements	Amt inj. daily	No. of tests	Total No. of chicks	% chicks w. tumors	Avg tumor size
None	—	6	60	3	+
Pteroylglutamic Acid	5 $\gamma$	3	23	61	+
" " "	10 $\gamma$	5	50	78	++
" " "	50 $\gamma$	6	57	88	+++
15 Unit Liver	.01 cc	1	10	70	+
15 " "	.025 cc	2	20	60	+
15 " "	.05 cc	2	20	60	++
15 " "	.10 cc	2	20	85	+++
Vit. B <sub>12</sub> concentrate	≡ .05 $\gamma$	2	20	55	+
" " "	≡ 0.1 $\gamma$	1	10	60	++
" " "	≡ 0.5 $\gamma$	2	20	75	+++
" " "	≡ 1.0 $\gamma$	2	19	68	+++
" " "	≡ 2.0 $\gamma$	2	20	85	+++
{ Vit. B <sub>12</sub>	{ ≡ 0.5 $\gamma$	1	10	90	++++
{ Pteroylglutamic Acid	{ ≡ 10 $\gamma$				
{ Vit. B <sub>12</sub>	{ ≡ 2.0 $\gamma$	1	10	100	++++
{ Pteroylglutamic Acid	{ ≡ 10 $\gamma$				

the synthetic pteroylglutamic acid deficient diet and water *ad libitum* and were injected with tumor suspension as previously described.<sup>2</sup> Supplements of pteroylglutamic acid and the preparations reported here were injected intramuscularly daily at the levels indicated in the table.

It was found that 15 unit liver extract\* would apparently replace pteroylglutamic acid at levels as low as 0.01 ml per day in stimulating the growth of Rous sarcoma in chicks fed this synthetic diet. It is known that parenteral liver extracts contain very little pteroylglutamic acid<sup>3</sup> and therefore cannot substitute for this factor in the nutrition of chicks. A preliminary study was then made of the properties of the factor in liver which was responsible for tumor stimulation.

Since 15 unit liver extract (beef) is a rich source of vitamin B<sub>12</sub> and the properties of the liver factor were found to be similar to this vitamin, highly purified concentrates of vitamin B<sub>12</sub> were also tested. These were found to give the same results as the liver extract itself. The preparations were previously assayed microbiologically<sup>4</sup> and by a

chick growth method<sup>4</sup> for their vitamin B<sub>12</sub> content.

Some typical results are presented in Table I. Pteroylglutamic acid alone gives a high incidence of growing tumors of good size. The liver extract alone gives quite similar results. The effect of the liver is duplicated by dosages of vitamin B<sub>12</sub> as low as 0.05  $\gamma$  per day. The higher levels of this factor give larger sized tumors than the lowest levels in the 14-day test period. When pteroylglutamic acid and vitamin B<sub>12</sub> are given together, the tumors come up earlier and attain greater size than with corresponding levels of either factor alone.

Thus it appears that vitamin B<sub>12</sub> does not entirely replace pteroylglutamic acid in its growth promoting effect on the Rous tumor but is required in addition to give maximum tumor growth under these particular dietary conditions.

*Summary.* Vitamin B<sub>12</sub> has been demonstrated to have a role in the growth of Rous sarcoma in chickens comparable to that previously shown for pteroylglutamic acid.

\* We thank Mr. A. C. Dornbush for these assays.

<sup>4</sup> Whitehill, A. R., Wieland, O., and Oleson, J. J., unpublished data.

\* 15 Unit liver-Lederle (prepared from beef liver).

<sup>3</sup> Clark, G. W., *Am. J. Med. Sci.*, 1945, **209**, 520.

6 hours) paralleled that produced by cortical hormone. The percentage of circulating eosinophiles dropped from 14.3 to 2.4, the per cent of lymphocytes remaining unaltered.

The absolute number of eosinophiles dropped from 2700 to 400 cell/mm<sup>3</sup>. In 2 adrenalectomized trichinous mice which received epinephrine, the total count had doubled within 6 hours. The percentages of eosinophiles and the other types of cells remained the same.

Urethane in aqueous solution (1 mg or ½ mg per g body weight) was injected into 6 intact trichinous mice. After 6 hours the absolute number of lymphocytes had dropped to approximately half, the eosinophiles to less than half the initial levels. Much greater decreases were obtained in 6 mice which survived 5 or 6 daily anesthetic doses of urethane. The percentage of eosinophiles dropped from 9.3 to 2.3, the absolute number from 1800 to 100. The absolute number of lymphocytes dropped from 12600 to 2200.

Urethane-induced lymphopenia has been observed in adrenalectomized as well as intact rats and mice.<sup>4,5</sup> Urethane depressed the white blood cell count in mouse myeloid leukemia, but adrenal cortical extract was ineffective.<sup>6</sup> These observations indicate that

the effect of urethane on normal blood lymphocytes and leukemic myeloid cells is not mediated by the adrenal cortex. In the present experiments eosinopenia paralleled lymphopenia following the injection of urethane. Adrenalectomized trichinous mice did not tolerate anesthetic doses of urethane, and in these experiments it could not be determined whether depression of blood eosinophiles occurs as an effect of urethane in the absence of the adrenal cortex. Spink<sup>7</sup> demonstrated that reduction in the eosinophile count of trichinous guinea pigs occurred with a superimposed bacterial infection. The eosinophile count was also suppressed in a patient developing bacterial infection during the course of trichinosis.

*Summary.* The number of circulating eosinophiles of trichinous mice was decidedly reduced by the administration of cortical hormone. The same effect was induced by epinephrine in intact, but not in adrenalectomized, trichinous mice. Sex steroids in the doses used did not significantly reduce the percentage of circulating eosinophiles in 4 intact and 2 adrenalectomized trichinous animals. Eosinopenia was induced by urethane in trichinous mice. Whether or not urethane-induced eosinopenia is mediated by the adrenal cortex has not been determined.

<sup>4</sup> Dury, A., and Robin, E. D., *Endocrinology*, 1948, **42**, 320.

<sup>5</sup> Lu, C. S., Thesis, University of Minnesota, 1947.

<sup>6</sup> Lu, C. S., Kirschbaum, A., *Proc. Internat. Cancer Res. Congress*, 1947, 105.

<sup>7</sup> Spink, W. W., *Arch. Int. Med.*, 1943, **54**, 805.

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### 17143. Effect of Pteroylglutamic Acid and Vitamin B<sub>12</sub> on Growth of Rous Tumor Implants.

J. J. OLESON AND PAUL A. LITTLE. (Introduced by Guy W. Clark.)

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Earlier work from this laboratory showed that pteroylglutamic acid was essential for the growth of implants of Rous sarcoma in young chicks.<sup>1</sup> The effect of other dietary

factors was much less pronounced.<sup>2</sup> A search was then made for additional dietary factors which might affect the growth of this sarcoma in chicks.

Day-old chicks in groups of 10 were fed

<sup>1</sup> Little, P. A., Sampath, A., Paganelli, V., Locke, E., and Subbarow, Y., *Tr. N. Y. Acad. Sci.*, Series II, 1948, **10**, 91.

<sup>2</sup> Little, P. A., Oleson, J. J., and Subbarow, Y., *J. Lab. and Clin. Med.*, 1948, **33**, 1139.

TABLE I.

Ovarian Weights in Immature Rats Injected with Extracts of Urine from Postmenopausal Patients. Aliquots of Pooled Specimens Were Adjusted to Different Degrees of Acidity Before Adsorption on Kaolin.

Exp. No.	Control Alcohol ppt.	Adsorbed on Kaolin at pH								Eluted with
		7.0	6.0	5.5	5.0	4.5	4.0	3.5	3.0	
1	69				59		19		46	3N NH <sub>4</sub> OH
	47				58		15		35	
2	—				99		57		17	"
	—				76		73		9	
3	136				112		101		81	2N NH <sub>4</sub> OH
	118				82		145		107	
4	—			16	87	72	58	65		"
	—			19	62	87	66	71		
5	—	18	18		32		39		20	"
	—	15	17		46		34		18	

Exp. 1, 2, and 3, urine acidified with hydrochloric acid.

Exp. 4 and 5, urine acidified with acetic acid.

Bring the clarified supernatant to pH 5.5 with glacial acetic acid.

7. Add 4 volumes of 95% ethanol, slowly and with constant stirring to precipitate the hormone. Set in refrigerator until thoroughly chilled, centrifuge and dry the precipitate in a gentle stream of air.

8. Dissolve the precipitate in a volume of water or phosphate buffer (pH 8.0) suitable for assay.

In our experience, urines from postmenopausal women or from patients with other hypergonadotrophic conditions give suitable assay dose ranges in infantile female rats if the final extract is made up so that 1 cc is equivalent to one hour's output of urine. Six injections of 0.5 cc each make the total dose equal to a 3 hour urine volume. The examples given below were made up in this way and show the ovarian weights at autopsy 72 hours after the first injection. If mice are used for assay the total dose can be reduced to the equivalent of about one hour's urine output. Assays to determine the quantities of hormone present in the urine of normal men or women require the use of larger quantities per test mouse or rat so the total dose should be equivalent to a 6 or 12 hour volume.

*Proof of method.* In the course of developing and testing the above method of adsorbing urinary gonadotrophins on kaolin, the optimal

conditions and quantities to be used in each step were determined. Usually the yield of hormone was compared to that obtained from an aliquot precipitated with alcohol as a standard of reference.

It has been customary to use fresh urine specimens to avoid partial or total destruction of the hormone through bacterial or chemical degradation. In three experiments the urine was allowed to stand several days and become very alkaline through the breakdown of urea to ammonia. When precipitated directly with alcohol these specimens yielded such highly toxic extracts that assays were not possible. After acidification and adsorption on kaolin, washing with water definitely reduced the toxicity of the extracts and hormone was recovered. In order to learn whether urease would affect the yield of hormone, that enzyme was added to urine and allowed to react for 2 hours with formation of ammonia. Subsequent acidification and adsorption on kaolin resulted in quantitative recovery of the hormone. The alkaline change due to urease activity is apparently not immediately destructive of the hormone. Collection of urine under toluene tends to preserve the natural acidity and the toluene is easily separated before kaolin is added.

The optimal pH for adsorption on kaolin was determined in several trials in which ali-

## 17144. Adsorption of Urinary Gonadotrophins on Kaolin.

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Zondek<sup>1</sup> described a method of obtaining gonadotrophic hormones from urine by precipitation with 4 volumes of alcohol. With minor modifications, such as the substitution of acetone for alcohol, this has remained the basic method of preparing urinary gonadotrophic hormones for assay.<sup>2-5</sup> Other procedures have been less commonly employed. Katzman and Doisy<sup>6</sup> recovered the hormone from urine by precipitation with acetone saturated with benzoic acid. Since the hormone is a protein, precipitating agents such as tannic acid<sup>7</sup> and ammonium sulphate<sup>8</sup> have been used, and ultrafiltration<sup>9,10</sup> has also proved successful. Katzman, *et al.*,<sup>11</sup> demonstrated that chorionic hormone could be adsorbed from urine by passing it through a column of permutit. Scott<sup>12</sup> and later Landgrebe and Samson<sup>13</sup> used kaolin as the adsorbing agent for chorionic hormone.

Since chorionic hormone could be adsorbed on permutit or kaolin it seemed probable that the pituitary gonadotrophin could also be

recovered from urine by an adsorption technique. This report describes a procedure by which either chorionic or pituitary gonadotrophins can be recovered from urine. The main advantages are, one, a great saving in alcohol over that which has been used previously and, two, a shorter time for preparation since dialysis is not necessary.

*Materials and method.* Kaolin (N.F. powder), that has been washed with normal hydrochloric acid and then with water until neutral, is made up as a 20% suspension in water. This is a stable stock preparation that should be shaken thoroughly before using.

1. Use a fresh 12 or 24 hour specimen of urine. Acidify to pH 4.5 with acetic (or hydrochloric) acid and then run through a coarse filter to remove mucus or particulate matter.

2. Add 5 volumes percent of the kaolin suspension to the acidified urine and stir thoroughly. Allow the kaolin to settle out. (One hour is usually sufficient but the mixture may be set in the refrigerator overnight).

3. Decant the supernatant and collect the sediment in a large centrifuge flask. Centrifuge, and discard the supernatant.

4. Add water and resuspend the kaolin by vigorous stirring or shaking. Centrifuge or allow to settle, decant and wash again with water until the kaolin is free of urine. This washing replaces the dialysis of the alcohol method.

5. Elute the hormone from kaolin by adding a volume of normal ammonium hydroxide equal to that of the kaolin suspension originally added to the urine. Stir thoroughly and allow to stand 20 to 30 minutes. Centrifuge and save the supernatant. The kaolin may be washed again, the second ammonia eluate added to the first and the kaolin discarded.

6. Add glacial acetic acid to the combined eluates until pH 8.5 is reached. If a flocculent precipitate forms, centrifuge and discard.

<sup>1</sup> Zondek, B., *Zentralbl. f. Gynak.*, 1929, **53**, 834.

<sup>2</sup> Bellerby, C. W., *Nature*, London, 1934, **133**, 494.

<sup>3</sup> Crew, F. A. E., *Brit. Med. J.*, 1939, 766.

<sup>4</sup> McCullagh, D. R., and Bowman, W. E., *Endocrinology*, 1940, **27**, 525.

<sup>5</sup> Heller, C. G., and Chandler, R. E., *J. Clin. Endocrinol.*, 1942, **2**, 252.

<sup>6</sup> Katzman, P. A., and Doisy, E. A., *J. Biol. Chem.*, 1932, **98**, 745.

<sup>7</sup> Levin, L., and Tyndale, H. H., *Proc. Soc. Exp. Biol. and Med.*, 1936, **34**, 516.

<sup>8</sup> Jones, G. E. S., and Bucher, N. L. R., *Endocrinology*, 1943, **32**, 46.

<sup>9</sup> Gorbman, A., *Endocrinology*, 1945, **37**, 177.

<sup>10</sup> Jungek, E. C., Maddock, W. O., and Heller, C. G., *J. Clin. Endocrinol.*, 1947, **7**, 1.

<sup>11</sup> Katzman, P. A., Godfrid, M., Cain, C. K., and Doisy, E. A., *J. Biol. Chem.*, 1943, **148**, 501.

<sup>12</sup> Scott, L. D., *Brit. J. Exp. Path.*, 1940, **21**, 320.

<sup>13</sup> Landgrebe, F. W., and Samson, L., *J. Obst. and Gyn. Brit. Emp.*, 1944, **51**, 133.

TABLE III.

Ovarian Weights of Immature Rats Injected with Extracts of Urine from Postmenopausal Patients. The amount of kaolin added to the urine was varied as indicated to determine the minimal amount of kaolin that would adsorb the hormone.

Exp. No.	Control Alcohol ppt.	Quantity of kaolin suspension Volumes %		
		10	3	1
9	—	130	107	140
		112	68	153
10	55	91	53	18
	64	63	49	18

the basis of these observations it seems that 5 to 10 volumes percent of kaolin suspension is best for routine use.

This method has been found equally applicable to recovery of chorionic hormone or of the pituitary hormone from the urine of postmenopausal women. In 2 experiments, these hormones were adsorbed simultaneously and the expected augmentation was demonstrated by assay of the final extract. One-half of a postmenopausal urine specimen was adsorbed on kaolin (a), while 100 cc of pregnancy urine was added to the other half before addition of the kaolin (a plus b). A second 100 cc portion of the pregnancy urine was also treated with kaolin (b). In the subsequent assays, the pituitary gonadotrophin (a) induced rat ovarian weights of 52 and 58 mg, whereas, those for the pregnancy urine extract (b) were 34 and 36 mg (with corpora lutea), and those of the combined urine eluate (a plus b) were 180 and 176 mg. This represented a definite augmentation reaction resulting from the simultaneous adsorption of the pituitary and chorionic gonadotrophins. Another example may be cited: a 19-year-old boy with infantilism associated with testicular agenesis had high urinary levels of pituitary gonadotrophins (kaolin extract of 6 hour urine volume induced ovarian weights of 52 and 58 mg). He was given chorionic gonadotrophin, and on the third day of treatment, excretion of the administered hormone together with the pituitary gonadotrophin was determined by assay. The same urinary equivalent of kaolin extract (6 hours output per test rat) produced ovarian weights of 257 and 260 mg.

This study was primarily a series of spot

checks to determine the limits within which a routine procedure could be developed. Small numbers of rats were used in each test since quantitative precision was not attempted. In 12 tests the extracts of alcohol precipitates were compared with those of kaolin adsorbates. In 3 instances material obtained by the alcohol method produced significantly greater increases in ovarian weights, in 6 trials the kaolin method seemed superior, and there was no significant difference in the other three. The sum of the ovarian weights for the 26 rats receiving extracts of alcohol precipitate was 2081 mg, as compared to 2073 mg for the 26 rats receiving kaolin extracts. Thus, there is no significant difference in the recovery of hormone by the two methods.

The main advantage of the kaolin method is the marked saving of ethanol. For example, the hormone from 1000 cc of urine can be adsorbed upon 50 cc of kaolin suspension, then eluted in two 50 cc portions of N ammonium hydroxide, which on subsequent acidification will not exceed 120 cc in volume and from which the hormone can be precipitated with 500 cc of alcohol. The original urine specimen would have required 4000 to 5000 cc of alcohol plus an additional quantity used in the second precipitation (after dialysis). A second advantage is that washing the kaolin with water accomplishes the same detoxification as dialysis but much more rapidly and avoids the transfer of solution to, and from, the dialysing bag.

Another advantage is that the same urine specimen can be used for both gonadotrophic and steroid determinations if the supernatant urine and the water washings from the kaolin are saved. A series of tests has shown that 17

TABLE II.

Ovarian Weights in Immature Rats Injected with Extracts of Urine from Postmenopausal Women. The hormone was adsorbed on kaolin and then the aliquots of the kaolin were eluted with different concentrations of ammonium hydroxide as noted.

Exp. No.	Control Alcohol ppt.	Eluted from kaolin with $\text{NH}_4\text{OH}$ Normality					No. of elutions
		3.0	2.5	2.0	1.5	1.0	
6	139	68		117		45	1
	128	41		113		15	
	148	106		92		16	
7	47	66		101	83	24	2
	43	82		71	90	19	
8		147	169	145	177	139	2 (After water wash)
		151	183	146	140	165	
		121	151	119	128	115	

quots of urine were adjusted to different degrees of acidity. In 3 experiments the pH was adjusted with hydrochloric acid (Table I) and recovery of the hormone was uniformly good at pH 5, but was poor in one instance at pH 4 and once at pH 3. In 2 other experiments using acetic acid the results were satisfactory from pH 3.5 to 5 but since the hormone was not adsorbed above or below this range, pH 4.5 has been selected as an optimal acidity for adsorption. Sulfuric acid was used in 2 experiments without success.

Having adsorbed the hormone onto kaolin, the next problem was to determine the optimal conditions for elution and recovery. Ammonium hydroxide was used in the elution process in concentrations up to 3 N without appreciable destruction or loss of hormone, (Table II). In Experiments 6 and 7 the recovery with 1 N  $\text{NH}_4\text{OH}$  was incomplete as compared to higher concentrations of ammonia but in Experiment 8 it was satisfactory. Experiments 6 and 7 were done before it was found that the kaolin can be washed with water and that the residual acid in the kaolin probably partially neutralizes the ammonia and reduces its efficiency. We have failed repeatedly to recover the hormone from kaolin with NaOH in concentrations from 0.1 N up to 3 N. Since elution with ammonia after using NaOH has also failed to recover the hormone, it appears that the pituitary gonadotrophin is easily and rapidly destroyed by sodium hydroxide. By contrast, chorionic gonadotrophin is apparently eluted quantitatively from kaolin by either 0.1 N NaOH or N  $\text{NH}_4\text{OH}$ .

Katzman, *et al.*, used 10% ammonium acetate in 40% ethanol to elute the chorionic hormone from permutit. This mixture is also satisfactory for eluting gonadotrophin from kaolin and is superior to ammonium hydroxide in alcohol, but, in our experience is not superior to aqueous ammonia. There are no experiments in which the dry weights of the final precipitate can be compared to give an indication of which eluting mixture may give the higher potency per mg of precipitate. The relative effectiveness of ammonium hydroxide in water and ammonium acetate in alcohol suggested that ethanolamine might be an effective elutant; this was proved in two experiments but seemed to offer no advantage over either of the others.

Other adsorbents were tried only casually. In three experiments the hormone was adsorbed on Filtercel and then recovered with ammonia. The coarser particles of permutit adsorbed the hormone more effectively when packed in a column and the urine allowed to percolate rather slowly. Charcoal (Norit) adsorbed the hormone but no eluting agent was found.

The quantity of kaolin necessary to provide a surface area adequate for complete adsorption and still have a minimal volume for elution procedures was determined in several experiments (Table III). In experiment 9, 1 volume percent (15 cc in 1500 cc of urine) of kaolin suspension gave as complete recovery of hormone as 10 volumes percent. However, in experiment 10, the smaller quantities of kaolin were less effective. On

agent, which was different, however, from streptomycin.<sup>4</sup> This difference may have been due to the fact that in the New Jersey collection the culture was kept on synthetic media and in the Baarn collection organic media are used. The difference pointed definitely to changes in the antibiotic-producing capacity during the 30-year period that the culture has been kept in the two collections.

Recently, Kelner<sup>6</sup> also examined the original *S. griseus* culture No. 3326. He confirmed the observation that the New Jersey strain is largely inactive antibiologically. When x-rayed conidia of this organism were plated out on various media and tested against bacteria, however, several active mutants were obtained, one of which apparently produced streptomycin. Comparison of the antibiotic spectrum of this mutant with that of known streptomycin-producing strains of *S. griseus* showed these spectra to be identical.

These observations appeared to be so important that an effort was made to confirm them and to demonstrate definitely that the antibiotic produced by Kelner's mutant No. 1 was actually streptomycin. This mutant, designated as 3326b, was streaked out on plates containing suitable agar media and, after 24- and 48-hour incubation periods, various test bacteria were cross-streaked toward it. An antibiotic spectrum characteristic of streptomycin-producing strains of *S. griseus* was obtained, as shown in Table I. There was only limited growth of the strepto-

mycin-resistant strains of *E. coli*, and there was good growth of streptomycin-dependent strains. This fully confirmed the streptomycin-producing capacity of Kelner's mutant. The presence of a narrow zone of inhibition of the streptomycin-dependent culture points to the formation of another antibiotic other than streptomycin, a fact well established for the various streptomycin-producing cultures.

To confirm definitely the fact that the antibiotic produced by the mutant of the 1915 culture was streptomycin, it was necessary to produce this antibiotic in suitable culture media, isolate it, concentrate it and test it. This was carried out by the usual procedures developed for streptomycin.<sup>1</sup> The results presented in Table II prove definitely that the antibiotic produced by the mutant of the 1915 culture (3326b) is identical with that of the 1943 isolate of the streptomycin-producing culture (3463). When varying amounts of the metabolite solutions of the two cultures were added to agar media and the latter streaked with various test bacteria, similar results were obtained, except for some minor quantitative differences. After 5 days' incubation a cup assay of 111 µg of streptomycin for the 3326b culture gave 150 *E. coli* dilution units, 250 *B. mycoides* units, 150 *S. aureus* units and 1,500 *B. subtilis* units; the corresponding results for 110 cup assay units for 3463 were 150, 500, 250 and 1,000, which were very close to the foregoing if one recognizes the limitations of this method of testing. The isolated and concentrated streptomycin preparations obtained from the two cultures also gave nearly identical results.

When streptomycin-dependent strains of *E. coli* were streaked on media containing the two preparations, comparable growth was obtained from both preparations and was very similar to growth with similar amounts of pure streptomycin added to other plates. This was true of both the culture filtrates and the concentrates obtained from them. The dependent strains of *E. coli* were affected alike by both preparations, whereas the resistant strains were not affected at all by either.

Other supporting evidence bearing upon the

TABLE I.  
Antibiotic Spectrum of Mutant 1 of 1915 *S. griseus* Culture (3326b), as Determined by Agar Cross-streak Method.

Zone of inhibition in millimeters.

Test organism	Nutrient agar	Nutrient glucose agar
<i>Escherichia coli</i>	24	19
" " sr*	3	7
" " sd*	6-25†	7-22†
<i>Bacillus mycoides</i>	24	21
<i>Staphylococcus aureus</i>	22	15
<i>Bacillus subtilis</i>	28	23

\*sr—streptomycin-resistant; sd—streptomycin-dependent.

†First figure—inhibition zone; second figure—growth zone.

<sup>6</sup> Kelner, A., *J. Bact.*, 1949, 57, 73.



ketosteroid estimations after the use of kaolin are the same as those obtained from untreated aliquots of urine.

**Summary.** A method is described for the adsorption of gonadotrophic hormones from urine onto kaolin and their subsequent elution with ammonium hydroxide. The relatively small volume of the eluate reduces the amount

of alcohol needed to precipitate the hormone from solution, the final extracts are less toxic and can be prepared in a shorter time. The hormone assays are comparable to those obtained with the usual alcohol precipitation method.

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### 17145. Streptomycin-Producing Capacity of Different Strains of *Streptomyces griseus*.<sup>\*†</sup>

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Since the first announcement<sup>1</sup> that certain strains of *Streptomyces griseus* produce the antibiotic streptomycin, pertinent questions have been raised in regard to the ability of this organism to produce the particular antibiotic: 1. Is this a characteristic property of all strains of the species *S. griseus*? 2. How widely distributed is the capacity for production of streptomycin among actinomycetes? 3. Did the original strain of *S. griseus*, first isolated in this country in 1915 by Waksman and Curtis,<sup>2</sup> also possess the capacity for producing streptomycin? (The last question is of considerable theoretical and historical interest, since it has a bearing upon the identity of the streptomycin-producing strains isolated in 1943 with the original 1915 isolate, as well as upon the fact that had the subject of antibiotics been recognized at that time, it would not have taken that long to recognize the actinomycetes as highly important producers of antimicrobial agents.)

The first two questions were soon answered.

<sup>\*</sup> Journal Series Paper, New Jersey Agricultural Experiment Station, Rutgers University. The State University of New Jersey, Department of Microbiology.

<sup>†</sup> Partly supported by a grant made by the Rutgers Research and Endowment Foundation.

<sup>1</sup> Schatz, A., Bugie, E., and Waksman, S. A., *Proc. Soc. Exp. Biol. and Med.*, 1944, **55**, 66.

<sup>2</sup> Waksman, S. A., and Curtis, R. E., *Soil Sci.*, 1916, **1**, 99.

It was established<sup>3</sup> that only certain few strains of *S. griseus* are capable of producing streptomycin, whereas most of the other strains that can easily be isolated from natural substrates are either unable to produce any antibiotics at all or may give rise to antibiotics other than streptomycin, such as grisein,<sup>4</sup> and that certain actinomycetes, other than *S. griseus*, also possess the capacity of forming streptomycin.<sup>5</sup>

In an attempt to answer the third question, the original 1915 culture, which has been kept alive by frequent transfers since its isolation in the collection of the New Jersey laboratories under the ATCC number 3326, was tested for its antibiotic-producing capacity. The same culture was deposited in 1920 in the Baarn collection in Holland; it recently was obtained from that collection and given the number 3326a. Repeated tests for their antibiotic-producing capacity revealed the fact that the culture kept in New Jersey was totally unable to produce either streptomycin or any other antibiotic substance, whereas the corresponding Baarn culture was capable of forming an antibiotic

<sup>3</sup> Carvajal, F., *Mycologia*, 1946, **38**, 596; Waksman, S. A., Reilly, H. C., and Johnstone, D., *J. Bact.*, 1946, **52**, 393.

<sup>4</sup> Waksman, S. A., Reilly, H. C., and Harris, D. A., *J. Bact.*, 1948, **56**, 259.

<sup>5</sup> Johnstone, D. B., and Waksman, S. A., *J. Bact.*, 1948, **55**, 317.

TABLE III.

Effect of Actinophage of Streptomycin-producing *S. griseus* on Various Strains of This Organism.

Culture No.	Nature of strain	Spot test* for phage		Submerged growth effect† Phage per ml $\times 10^7$ , after days of incubation			
		Nutrient agar	Dextrose-asparagine agar	0	3	5	7
3326	N. J. culture of 1915 isolate	—	—	6	3	4	4
3326a	Holland culture of 1915 isolate	—	—	6	<0.4	<0.04	0.004
3326b	Kelner's mutant of 1915 isolate	+	+	6	100	105	186
3463	Streptomycin-producing soil culture of 1943	+	+	6	30	31	119
3464	Streptomycin-producing chicken throat culture of 1943 isolate	+	+	6	210	320	570
3464a	Spore isolate of last	+	+	6	34	260	204
3478	Grisein-producing culture	—	—	6	2	2	3
3481	More recent independent isolate of a streptomycin-producing culture	+	+	6	257	420	400
3482	Non-streptomycin-producing culture	—	—	6	0.2	0.01	0.03

\* Phage placed on 24-hour vegetative streaks growing on nutrient and dextrose-asparagine agars, incubated an additional 24 hours, and examined for lysis.

† Phage added to freshly inoculated flasks of medium, grown under submerged conditions.

the culture upon artificial media for more than 30 years. When irradiated, this culture yielded a mutant that was as potent a producer of streptomycin as the streptomycin-yielding strains of *S. griseus* isolated in 1943.

This assumption was fully confirmed by the characteristic antibiotic spectrum of the two cultures, their behavior to streptomycin-dependent and streptomycin-resistant mutants of *E. coli* and other bacteria, and sensitivity of the two cultures to the same actinophage.

These results further point to the accuracy of the identification of the streptomycin-pro-

ducing cultures of *S. griseus* with that of the original 1915 isolate, based on literature description alone, in spite of the fact that the type culture available in two collections showed distinct changes from the original description.

The authors wish to express their sincere appreciation to Dr. A. Kelner of Cold Spring Harbor for placing the mutant of *S. griseus* at their disposal, and to Mr. W. Iverson of this laboratory for assisting with some of the tests.

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## 17146. Effect of Tetraethylammonium Bromide on Adrenal Medulla.

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Acheson and Moe<sup>1</sup> have shown that tetraethylammonium ion will block autonomic ganglia. Since the adrenal medulla is generally accepted as ganglionic in nature, the transmission of impulses across the ganglion and subsequent release of epinephrine should be blocked by the injection of tetraethylammon-

ium bromide. Some evidence for this has been shown by the action of tetraethylammonium on morphine hyperglycemia in dogs,<sup>2</sup> which closely resembles the effect on such hyperglycemia produced by surgical removal of the adrenal medulla.<sup>3</sup> In both cases mor-

<sup>2</sup> Morrison, J. L., *Fed. Proc.*, 1947, 6, 359.

<sup>3</sup> Bodo, R. C., Co Tui, F. W., and Benaglia, A. E., *J. Pharm. and Exp. Therap.*, 1931, 61, 48.

<sup>1</sup> Acheson, G. H., and Moe, G. K., *J. Pharm. and Exp. Therap.*, 1946, 87, 220.

TABLE II.

Identity of Antibiotic Produced by Culture 3326b with That of Streptomycin Produced by Known Streptomycin-producing Culture 3463.

Incubation, days	Streptomycin, μg/ml	Dilution units per ml			
		<i>E. coli</i>	<i>B. mycoides</i>	<i>S. aureus</i>	<i>B. subtilis</i>
		Culture filtrate of 1915 mutant (3326b)			
3	59	75	150	75	500
4	108	100	250	250	750
5	111	150	250	150	1,500
		Crude isolated preparation of 3326b			
	867*	1,500	3,000	1,500	>10,000
		Culture filtrate of streptomycin-producing culture (3463)			
3	41	30	100	75	300
4	64	75	150	75	750
5	110	150	500	250	1,000
		Crude isolated preparation of 3463			
	770*	1,000	3,000	500	10,000

\* Culture filtrate adsorbed on Norite, dried, eluted with acid alcohol, treated with ether, to yield aqueous concentrate.

problem under consideration may be reported here. It had been suggested previously<sup>7</sup> that the sensitivity of streptomycin-producing strains of *S. griseus* to a specific actinophage could be used for establishing the capacity of such strains to form streptomycin. The original 1915 culture of *S. griseus* (3326) was not sensitive to this particular phage; this was taken to indicate that there was a definite correlation between phage sensitivity of *S. griseus* strains and ability to form streptomycin, a fact confirmed by a lack of sensitivity to phage by other, freshly isolated, non-streptomycin-producing strains of *S. griseus*. When Kelner's mutant was tested for sensitivity to this phage, it was found to be highly sensitive. This is illustrated in Table III. The streptomycin-producing, including the two original isolates (3463, 3464), a spore isolate of one of these (3464a), and a freshly isolated culture (3481), were all phage-sensitive as measured by the rate of cell multiplication. Both forms of the original 1915 isolate (3326, 3326a), a grisein-producing strain of *S. griseus* (3478), and another non-streptomycin-producing culture (3482) were all insensitive to this phage, as shown by lack of multiplication or by actual adsorption by the mycelium of the organism. On the other hand, the Kelner mutant of the 1915 isolate

(3326b) was phage-sensitive.

The identical behavior of the mutant of the 1915 culture with that of the streptomycin-producing strains of *S. griseus* is thus established unequivocally. Other evidence of similar cultural characteristics, sporulation, and biochemical properties further confirmed the identity of the two cultures.

These results thus suggest the probability that the 1915 isolate of *S. griseus* (3326) possessed originally the capacity of producing streptomycin, and that this capacity was lost during the more than 30 years that this culture was kept in the culture collection, growing on artificial, mostly synthetic, media. The ability of streptomycin-producing *S. griseus* cultures to mutate readily, even under ordinary conditions of culture, has been well established.<sup>8</sup> At least two mutants are now recognized, one of which does not sporulate and does not produce streptomycin, and one of which produces a red vegetative mycelium and forms an antibiotic substance distinct in nature from streptomycin.<sup>4</sup>

**Summary.** Evidence is presented that the original culture of *S. griseus* which was isolated from different soils, in 1915-1916, in the laboratories of the New Jersey Agricultural Experiment Station must have possessed the capacity for producing streptomycin. This capacity was lost upon continuous growth of

<sup>7</sup> Waksman, S. A., Reilly, H. C., and Harris, D. A., *Proc. Soc. Exp. Biol. and Med.*, 1947, **66**, 617.

<sup>8</sup> Schatz, A., and Waksman, S. A., *Proc. Nat. Acad. Sci.*, 1945, **31**, 129.

num effects of tetraethylammonium indicated that tetraethylammonium had no demonstrable effect on the action of epinephrine at the effector cells of the nictitating membrane.

**Results.** Tracings illustrating a typical experiment in Fig. 1 show that tetraethylammonium compound blocks the release of epinephrine from the adrenal medulla.

In all of the 6 cats used in this study the nictitating membrane was stimulated to contract as a result of electrical stimulation of the splanchnic nerve. Repetition of stimuli at intervals over a period of 30 minutes to one hour produced similar contractions. Retraction usually persisted for several minutes.

After administration of tetraethylammonium bromide, stimulation of the splanchnic nerve was not followed by retraction of the membrane. Injection of 3 mg/kg abolished retraction in most of the animals but 10 mg/kg abolished it in all animals. However, intravenous injection of .001 mg/kg of epinephrine hydrochloride at the peak of the tetraethylammonium effect produced marked retraction of the nictitating membrane.

**Summary.** 1. Tetraethylammonium bromide blocks the release of epinephrine from the adrenal medulla.

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### 17147. Tumorous Growths in the Pituitary and Trachea Following Radio-toxic Dosages of $I^{131}$ .\*

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The possible occurrence of thyroidal tumors after partial beta-ray destruction of the thyroid gland has prompted the investigation of long-term effects of high dosages of  $I^{131}$ . No thyroidal growths have been observed in our experiments with mice, but tumors of the trachea and hypophysis have been found. This report deals with the incidence of such tumors and provides a description of the growths as well as the conditions under which they are obtained. A detailed characterization of the cytological properties of the hypophyseal tumors is being prepared separately.

**Methods and materials.** Most of the work included in this report was done with adult mice of the  $C_{57}$  strain. Smaller numbers of the A,<sup>1</sup> I<sup>1</sup> and CFW<sup>2</sup> strains also were em-

ployed. Although more than 500 animals have been utilized in different phases of this study, the present report concerns 158 mice observed for a period of time sufficient to show the metaplastic and atypical growth changes, as well as 34 untreated control mice. Purina Laboratory Chow and water comprised the exclusive *ad libitum* diet of the mice, and they were kept at controlled temperature ( $72^{\circ} \pm 2^{\circ}F$ ), humidity 65% ( $\pm 5\%$ ), and illumination (12 hours per day).

Radioactive iodine was given subcutaneously as NaI without carrier, in neutral aqueous solution in dosages of 10, 50, 80, 100, 160, 200, 300, 400, 500 and 1000 microcuries.<sup>3</sup> At least 10 animals were included in each dosage group for the  $C_{57}$  and CFW strains, and in most cases, especially with the 100, 200 and 300 microcurie dosages, there were 40 or more animals.

Calibration and measurement of the dosages was done with a Geiger counter and standards

<sup>3</sup> 100  $\mu c$  in normal adults produces almost complete, and 200  $\mu c$  complete, thyroidal destruction.

<sup>1</sup> Gorbman, A., *Proc. Soc. Exp. Biol. and Med.*, 1947, 66, 212.

\* This study has been supported in part by the National Cancer Institute, U. S. Public Health Service, and in part through contract with the Atomic Energy Commission and Office of Naval Research.

<sup>2</sup> Breeding stock of these strains originally obtained from colony of L. C. Strong, Yale University.

<sup>3</sup> Purchased from Carworth Farms, New City, N. Y.

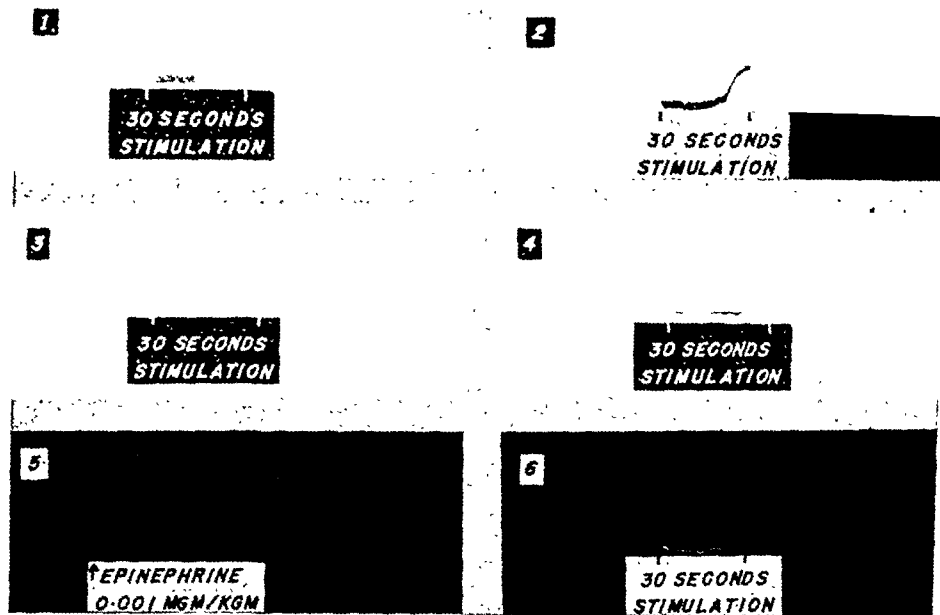


FIG. 1.

Cat C. Effect of stimulation of splanchnic nerve on retraction of nictitating membrane. (1) Initial stimulation in anesthetized cat. (2) Thirty minutes after first stimulation. (3) Ten minutes after tetraethylammonium bromide 10 mg/kg I.M. (4) Thirty minutes after injection of tetraethylammonium bromide. (5) Effect of epinephrine hydrochloride I.V. 45 minutes after tetraethylammonium bromide. (6) Seventy-five minutes after I.M. injection of tetraethylammonium bromide.

phine hyperglycemia is abolished or greatly diminished. A more direct method of determining blockade of the adrenal medulla is desirable than shown by blood sugar levels. Sensitivity of the denervated nictitating membranes of cats to circulating epinephrine offers a convenient biological test of such a blockade.<sup>4</sup>

**Method.** In a series of 6 cats the superior cervical ganglion on the left side was removed. During a recovery period of 4 days the nictitating membrane becomes sensitized to circulating or injected epinephrine. The cats were anesthetized and the following preparations made. Through an incision in the body wall, shielded electrodes were placed over the splanchnic nerve just before it enters the adrenal gland in order to provide for stimulation. The eyelids were retracted after cutting the lateral canthus and the nictitating membrane was connected to an isotonic lever mag-

nifying its movement several times and recording them on a revolving smoked drum. Splanchnic nerves were stimulated using a Bird Stimulator before and after intramuscular injection of a 10% solution of tetraethylammonium bromide. In all cases after full effect of the tetraethylammonium bromide had been shown, epinephrine was injected in order to demonstrate the action of epinephrine on the nictitating membranes. Either dial solution\* or pentobarbital sodium was used as the anesthetic. We could find no appreciable difference in the response of the animals to these barbiturates. Doses of tetraethylammonium bromide ranged from 3 mg/kg up to 10 mg/kg. Maximum action was obtained in 10 to 15 minutes and lasted for about an hour after which time it began to wear off. Intravenous injections of varying amounts of epinephrine hydrochloride at any time during the maxi-

\* Dial Solution furnished through courtesy of Dr. Frederick F. Yonkman, Ciba Pharmaceutical Products, Inc.

<sup>4</sup> Rosenblueth, A., and Cannon, W. B., *Am. J. Physiol.*, 1932, 99, 398.

was 34.7 mg. The data indicate a tendency for larger tumors of earlier occurrence after the largest ( $300+\mu\text{c}$ ) dose. The 3 largest tumors weighed 242, 204 and 210 mg, and followed 325 and 200 and  $100\mu\text{c}$  dosages, respectively. Excluding 5 extremely large glands of over 150 mg, the average weights of pituitaries 250 days or more following injection, were 27.5 mg for the  $100\text{--}160\mu\text{c}$  dosage group, 39.7 mg for the  $220\text{--}225\mu\text{c}$  group, and 87.3 mg for the  $300+\mu\text{c}$  group.

The glands weighing more than 20 mg characteristically showed hemorrhagic areas, particularly on the dorsal side near the infundibular stalk. Gross examination of serial sections of the tumors proved that they are limited to the anterior lobe.

Pituitaries weighing more than 30 mg frequently produced blindness and motor disturbances in the mice. Such glands lost the smooth outlines of smaller tumors and frequently were lobed. In most instances a hollow was formed on the ventral side of the brain by displacement of its parts. The larger tumors, by pressure against the brain, actually caused various degrees of separation of the parietal and occipital bones. The resulting distortion in the shape of the head made it possible to identify, from external examination, those animals with tumorous pituitaries. All of the hypophyseal tumors appeared to exert their influence upon neighboring tissue by pressure alone. In no instance was there evidence of erosion or invasion of brain or bone or other tissue by growing hypophyseal masses.

*Trachea.* In contrast with most other tissues, the tracheal epithelium appeared extremely sensitive to the accumulated radiant energy in the thyroid gland, and cellular responses were visible as early as one day following  $\text{I}^{131}$  injection. Early responses were all of the degenerative type, and their degree and rate of development varied with the size of the  $\text{I}^{131}$  dose.

Pycnosis of nuclei, loss of cilia, and finally death and desquamation of the cells was the usual order of changes observed after dosages of  $80\mu\text{c}$  or more. Even with the lowest effective dose such changes were completed

within a week, and regeneration begun at that interval. The  $80\mu\text{c}$  dose produced minimal lesions; the  $1000\mu\text{c}$  dose produced within 48 hours complete desquamation of that tracheal epithelium closest to the thyroid. In cases of large scale loss of the tracheal epithelium accumulations of macrophages appeared within several days and were seen to contain cellular debris. After the  $80\text{--}160\mu\text{c}$  doses, tracheal epithelial regeneration was, as a rule, of the normal type and occurred from the normal epithelium surrounding the lesions. After doses of more than  $200\mu\text{c}$  tracheal regeneration was, as a rule, with a metaplastic epithelium of variable form and only rarely entirely normal. Sometimes it consisted of cuboidal ciliated or unciliated cells. Most often, especially with the higher doses, it was simple or stratified squamous in type. Some of the stratified squamous tracheal epithelia in CFV mice were extremely active mitotically.

Changes in the fibrous tunica propria and in the tracheal cartilages also occurred, but only with doses in excess of  $300\mu\text{c}$ . Temporary loss of basophilia in the cartilage often was observed soon after injection, and death of many chondrocytes was usual. Such changes often were followed by a hyaline thickening of the perichondrium which in some instances became as thick as the entire cartilage and in some instances strongly resembled bone. Thickening of the fibrous tracheal tunica propria was usual after the higher doses in animals sacrificed about six to ten months after injection. In 5 extreme cases the fibrous thickenings projected into the tracheal lumen as small tumors almost completely obstructing it. Such animals before autopsy had a labored wheezing type of breathing.

*Discussion.* The mechanism by which tumorous growth in pituitaries was elicited in our experiments does not seem to be deducible from the data at our disposal. Administration of goitrogens leads to mild enlargement of the pituitary gland.<sup>2</sup> In this laboratory (unpublished data) a 0.1% thiouracil diet fed to mice for almost 400 days yielded

<sup>2</sup> Higgins, G. M., *Am. J. Med. Sci.*, 1945, **210**, 347.

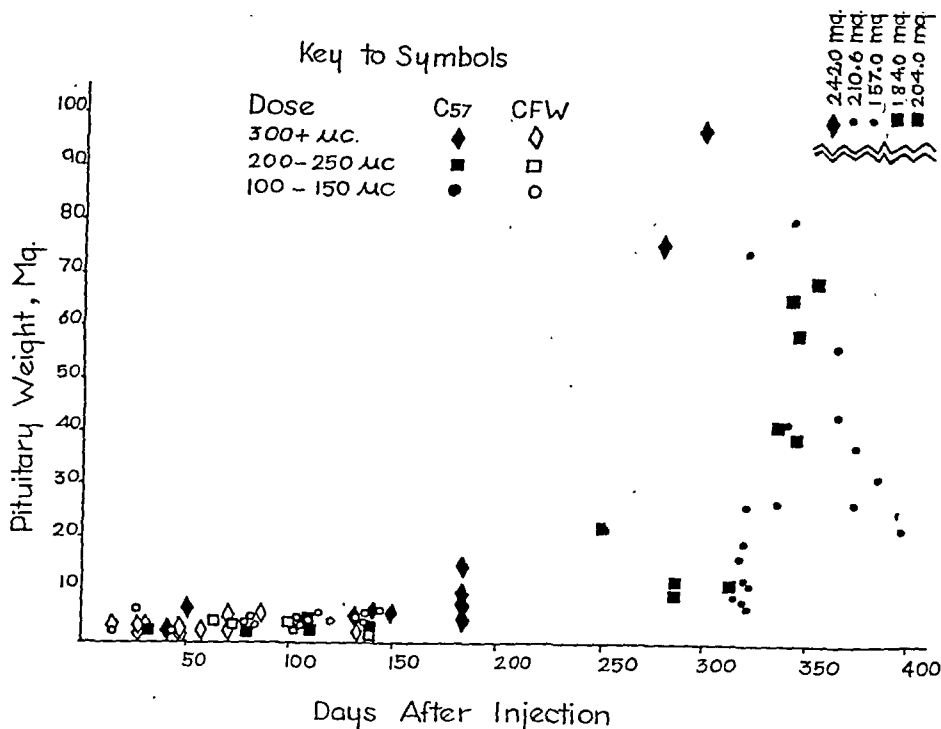


FIG. 1.  
Weights of pituitary glands after injection of  $I^{131}$ .

which consistently have given a millicurie unit with about 90% of the activity indicated by measurements of the Oak Ridge National Laboratory. Measurement of  $I^{131}$  uptake in several separate series of mice injected under these conditions showed that only 5% to 10% of the administered  $I^{131}$  is stored in the thyroid, reaching this maximum 12 to 14 hours after injection. The pituitary in our observations never contained  $I^{131}$  in concentrations significantly higher than the blood.

Animals were sacrificed in groups at intervals, or singly at irregular times if moribund. Pituitaries were fixed in 10% formalin and weighed from 70% alcohol. Tracheas were sectioned serially for microscopic examination.

**Observations.** The 10  $\mu$ C and 50  $\mu$ C dosages were without effect upon the pituitary, as judged by weight. The 80  $\mu$ C dose was similarly ineffective in producing tumorous pituitaries, although it did elicit a 3-fold or 4-fold enlargement of the gland in animals which were of small weight at the time of

injection. The 80  $\mu$ C dose also produced metaplastic changes in the tracheal epithelium. Dosages of 400  $\mu$ C or more almost always resulted in death at an interval too early for development of hypophyseal tumors. For these reasons detailed discussion of results is confined to animals given less than 400  $\mu$ C of  $I^{131}$ .

**Pituitary gland.** Fig. 1 indicates the general pattern of results obtained. It seems clear that soon after injection of 100  $\mu$ C to 325  $\mu$ C quantities of radioactive iodide, and up to 150 days thereafter, there was mild enlargement of the hypophysis. The average control pituitary weight in this interval was 1.2 mg. The average of  $I^{131}$ -injected animals was 3.34 mg. Between 150 and 250 days rapid increments in pituitary weights occurred in mice given adequate  $I^{131}$  dosage. As indicated in Fig. 1, after 250 days very few pituitaries under 10 mg could be found. The average control pituitary of older mice weighed 2.1 mg. The average of all the  $I^{131}$  mice

# 17148. Influence of Environment on Prewearing Growth of the Rat. I. Dietary Regimen of the Young.<sup>3</sup>

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Evidence presented in a previous publication<sup>2</sup> indicated that the growth curve of preweanling rats in the authors' laboratory did not conform to the formulation of preweaning growth given by Zucker *et al.*<sup>3,4</sup> It was found with the Anderson and Smith<sup>5</sup> diet that Zucker's equation,  $\log W = k \log t + \log C$ , applied to the approximate postconception age periods, 22 to 34 and 43 to 50 days, but that there were changes in the constants of this equation at fairly definite ages. The first change at approximately 24 days was believed to be correlated with the birth-weight but the second change at about 29 days of age was tentatively ascribed to a change in growth pattern. The divergence of the curve from a straight line at about 34 to 43 days was assumed to be due primarily to some dietary influence.

These results were obtained under the usual breeding conditions wherein the mother and young were housed in a single cage and all had access to the diet. It is generally recognized that the young begin to ingest solid food coincident with the opening of their eyes at about 34 days after conception and it has been suggested<sup>2</sup> that this phenomenon may be associated with the divergence of the growth curve. The following experiments, fashioned after those of Kozłowska *et al.*<sup>6</sup> except that

no mineral supplements were employed, were performed to elucidate the factors contributing to the observed divergence and to determine the pattern of growth when the young are restricted to the mother's milk.

*Experimental.* Seven pregnant Long-Evans strain rats were placed in individual breeding cages. The first group (3 rats) was fed the Anderson and Smith diet *ad libitum* in the breeding cages until the young were weaned. The second group (3 rats) was removed from the breeding cages (which contained no food) for 2-hour periods 3 to 4 times daily during the period 7:30 A.M. to 10:30 P.M. and allowed to eat the same diet *ad libitum* in separate cages. This procedure was continued until the young were weaned at 50 days postconception (28 days, post-natal). At this point the mother was removed from the breeding cage and the young were given the Anderson and Smith diet *ad libitum*. The third group (1 rat) was fed similarly except that separate feeding of the mother was discontinued at 43 days postconception and the diet was made available *ad libitum* in the breeding cage until the young were weaned at 50 days postconception.

Each of the litters was reduced at birth to 6 young divided, so far as possible, equally between males and females. The total weight of each sex in each group was determined daily and the total weights were divided by the number of males and females, respectively. The plot of the resultant averages is shown in the accompanying figures. These procedures afforded growth data on a total of 39 young (18, 15 and 6, respectively) in the 3 groups.

*Results.* The growth curves obtained for the male rats are shown in Fig. 1. Only the curves for the males are given inasmuch as the results were similar with the females. Since the growth data of the 10 males of

<sup>1</sup> Paper 60. For Paper 59 see Dunn *et al.*<sup>1</sup> The writers' work has been aided by grants from the Nutrition Foundation, Inc., and the University of California. The authors are indebted to Walter S. Trasin for technical assistance.

<sup>2</sup> Dunn, M. S., Camien, M. N., Eiduson, S., and Malin, R. B., in press.

<sup>3</sup> Murphy, E. A., and Dunn, M. S., *Growth*, 1948, 12, 311.

<sup>4</sup> Zucker, L., Hall, L., Young, M., and Zucker, T. F., *Growth*, 1941, 5, 399.

<sup>5</sup> Zucker, L., Hall, L., Young, M., and Zucker, T. F., *Growth*, 1941, 5, 415.

<sup>6</sup> Anderson, W. E., and Smith, A. H., *Am. J. Physiol.*, 1932, 100, 511.

<sup>7</sup> Kozłowska, C. M., McCay, C. M., and Maynard, L. A., *J. Nutr.*, 1932, 5, 61.



pituitary weights as high as 8.2 mg, and averaging 4.4 mg, compared to the control weight in old mice of 2.0 mg.

It does not seem likely, therefore, that the radiation thyroidectomy experienced by our animals is the principal factor to be considered, though it conceivably might play a synergistic role in combination with some other influence.

It has been well established<sup>3,4</sup> that chronic administration of estrogens to rodents results in tumorous enlargements of the pituitary. It is tempting to note that many of our long-term C<sup>57</sup> females were in extended periods of vaginal cornification, and had enlarged mammary glands and uteri, as well as loosening of the pubic symphysis, when sacrificed 300 or more days after treatment. On the other hand, male C<sub>57</sub> mice were found to have some of the largest hypophyseal tumors in the series and yet displayed none of the usual signs of estrogenization. At the present time, attempts are being made at experimental analysis of this situation, but a really rational approach to the problem does not seem to be indicated by the available data.

It would seem that the destruction of tracheal epithelium in our mice is a direct result of the ionizing beta radiation emanating from the thyroid gland. The regenerated epithelia, though usually metaplastic never showed neoplastic tendencies, excepting possibly those cases of stratified squamous epithelia which were mitotically extremely active. Though apparently benign, the tracheal

tumors found in our mice were, or were about to be, fatal through tracheal obstruction and hindrance to respiration.

It should be emphasized that the range of beta particles of I<sup>131</sup> in tissue is usually considered to be approximately 2 mm.<sup>5</sup> The thyroid capsule alone is as thick as this around most human thyroids, and hence beta irradiation of thyroid-contiguous tracheal structures appears unlikely. Nevertheless, attention has been drawn<sup>6</sup> to the relatively frequent occurrence of signs of tracheal injury in patients undergoing I<sup>131</sup> therapy for hyperthyroidism. The possibility of fibrous tracheal proliferation in humans following I<sup>131</sup> treatment of the thyroid remains to be considered.

*Summary.* Following doses of radioactive iodine sufficient to cause destruction of most or all of the thyroid, tumorous enlargements were found in the pituitary glands and tracheas of mice. Hypophyseal growths as large as 240 milligrams (control, 2.1 mg) were found. Less than 10% of animals sacrificed more than 250 days after I<sup>131</sup> administration had pituitaries smaller than 10 mg in weight.

Tracheal tumors of the fibrous tunica propria, obstructing the tracheal lumen, were of lower incidence (4%) but appeared earlier. Fibrous thickenings of the tunica propria, and metaplastic regeneration of tracheal epithelium were more common.

<sup>3</sup> Cramer, W., and Horning, E. S., *Lancet*, 1936, **230**, 1056.

<sup>4</sup> Wolfe, J. M., and Wright, A. W., *Endocrinology*, 1938, **23**, 200.

<sup>5</sup> Marinelli, L. F., Quimby, E. H., and Hine, G. J., *Am. J. Roentgenol. and Rad. Therap.*, 1948, **59**, 260.

<sup>6</sup> Lukens, R. M., *Ann. Otol. Rhinol. and Laryngol.*, 1948, **57**, 633.

Received May 6, 1949. P.S.E.B.M., 1949, **71**.

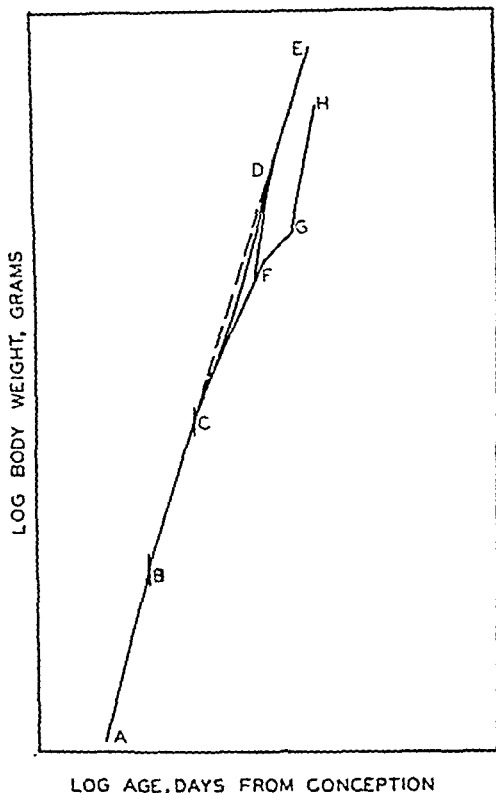


FIG. 2.

Superimposed logarithmic curves relating body weight and age of male Long-Evans strain rats under different dietary conditions.

Solid line, ABCDE—Curve 1 of Fig. 1. Unrestricted feeding.

Solid line, ABCFGH—Curve 2 of Fig. 1. Restricted, to 50 days.

Solid line, ABCFDE—Curve 3 of Fig. 1. Restricted, to 43 days.

lute size will be considered in a subsequent publication. In Fig. 2 the solid line, ABCDE, represents the growth of the males in Group 1 (unrestricted feeding); line ABCFDE represents the growth of the males in Group 3 (restricted to 43 days); and line ABCFGH represents the growth of the males in Group 2 (restricted to 50 days, weaning). It may be concluded from these results that the slopes of the segments CF and FG represent the rates of growth natural to the milk diet during these age periods and that the slope of segment DE is typical of the natural rate of growth on the solid diet after 43 days of age. The shape of the growth curve of the unrestricted males, therefore, would appear to be

the resultant of the natural transition from the milk diet (CF) to that of the completely solid diet (DE) with the completion of the physiological ability to utilize fully the solid diet occurring at about 43 days (postconception). In this case the curve is naturally concave. Since, in the Group 3 animals there is present the physiological capacity to utilize the solid food, the resultant convexity of the curve is indicative of the "recovery" nature of the growth. This reasoning also accounts for the increase in slope of GH over that of DE.

Since the data of Group 1 agree very well with other similar data obtained earlier and with different groups of animals, they have reasonable reliability. That segments of the 3 curves have the same relative shape and are superimposable is further evidence supporting this view. It would seem to be a logical conclusion that the divergence from a straight line between about 34 to 43 days (illustrated by Curve 1, Fig. 1 and reported previously<sup>2</sup>) does not indicate necessarily a qualitative deficiency in the diet but may be a natural phenomenon arising because of the transition from a milk diet to one of solid food. Although this explanation was suggested by MacDowell *et al.*,<sup>7</sup> the present experiments appear to be the first to demonstrate its validity. That the causes were quantitative, rather than qualitative, in nature was indicated in previous experiments<sup>2</sup> with a limited amount of data. It was also shown earlier that the degree of divergence and/or its duration could be influenced by the quality of the diet.

**Summary.** The shape of the preweaning growth curve of the rat is profoundly affected by the lack or availability of solid food. The divergence from conformity to Zucker's growth equation of rats raised on the Anderson and Smith diet may be due to the natural transition from a liquid (milk) to a solid diet and does not appear to be explained by a qualitative deficiency in this diet.

<sup>7</sup> MacDowell, E. C., Gates, W. H., and MacDowell, C. G., *J. Gen. Physiol.*, 1930, **13**, 529.

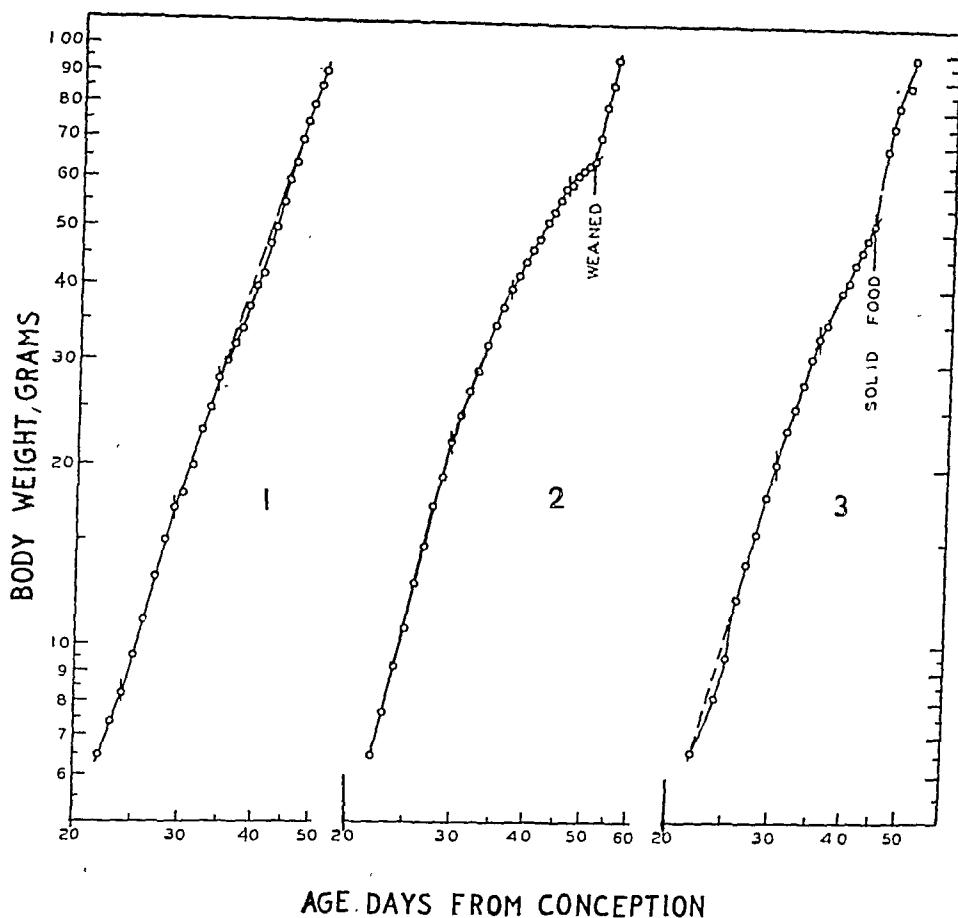


FIG. 1.

Logarithmic curves relating body weight and age of male Long-Evans strain rats under different dietary conditions.

Curve 1. Average of 33 rats, unrestricted feeding.

Curve 2. Average of 10 rats, restricted until weaning.

Curve 3. Average of 4 rats, restricted until 43 days of age.

Group 1 and those obtained previously were in close agreement the two sets of data were combined. The resultant curve (Curve 1, Fig. 1), therefore, represents the average growth of 33 males. The average curves representing the nine males of Group 2 and the 4 males of Group 3 are shown as Curves 2 and 3 of Fig. 1, respectively.

**Discussion.** It is apparent that the shape of the growth curve of the young, particularly from about 34 days of age (postconception) to weaning, is profoundly affected by the availability of solid food. The changes in slope of Curves 2 and 3 occurring between 34 days and weaning may reflect both the decreasing

food value to the young rat and the decreasing volume of the mother's milk.<sup>2</sup> The insufficiency of the milk as a food source with increasing size of the young rat is emphasized by the abrupt increase in growth rate as soon as solid food became available. The correlation of these data is shown in Fig. 2 which was constructed by superimposing the curves for the male rats given in Fig. 1. This was accomplished by aligning the tracings so that point B and the line BC on each of the 3 curves coincided. In this way the curves are plotted approximately identically on the X scale (age) without regard for the Y scale (weight). The effect of environment on abso-

TABLE I.  
Properties of Fractions Obtained by Electrophoresis of EW Inhibitor Preparation A200 PII EII in Phosphate Buffer of 0.1 Ionic Strength and pH 7.2.

Fraction	Vol. recovered (ml)	N ( $\gamma$ per ml)	Inhibition titer	Partition of total recovered, N (%)	Partition of total recovered, activity (%)	Purification factor*
Starting material		95.5	12,800			40
Descending limb	6.1	25.8	1,500	19	6.8	17
Ascending "	6.4	17.2	2,700	13	13	47
Bottom cap						
(a) supernatant	2.2	85.6	12,100	22	20	42
(b) precipitate†	1.3	298	62,000	46	60	62
(c) total‡	3.5	165	31,000	68	80	56
Crude EW		16,700	55,500			1.00

\* Calculated from the titer/N ratio with reference to the titer/N ratio of crude EW.

† An almost transparent gel. Deducting the nitrogen and inhibitory activity of an equal volume of supernatant, one obtains the value 71 for the purification factor of the solid phase.

‡ Calculated by combining the results for supernatant and precipitate.

phate buffer at pH 7.2, each at 0.1 ionic strength.<sup>1</sup> Inhibitory activity was titrated against 4 hemagglutinating doses of heated (53°C, 30 minutes) purified swine influenza virus.<sup>1</sup> Nitrogen was determined by direct nesslerization.

*Experimental.* The following experiment is illustrative. Semi-purified inhibitor, dialyzed against phosphate buffer, was submitted to electrophoresis at 5 volts per cm. Within 3 minutes, the whole solution, initially almost clear, acquired a ground-glass appearance. Floccules formed gradually and settled toward the bottom cap. Precipitate remaining in the descending limb after 3 hours is shown in Fig. 1d; settling was often complete in the ascending limb (Fig. 1a). The isolated contents of the 2 limbs and bottom cap, the latter after centrifugation for 10 minutes at 870 g, were analyzed for nitrogen and inhibitory activity. The results (Table I) show (a) concentration of inhibitory activity in the bottom cap; (b) association of the activity with the precipitate; and (c) purification of the inhibitor consequent to electrical precipitation.

Precipitation occurred frequently during electrophoresis of solutions of whole or thick EW, with results analogous to those of Table I. The precipitation seemed more pronounced in veronal than in phosphate buffer and could

† Precipitate containing inhibitory activity frequently developed during dialysis of 25% whole or thick EW and was removed by centrifugation prior to electrophoresis.

be prevented in the former by vigorous blending without significant decrease in inhibitory activity. With semi-purified inhibitor, vigorous blending delayed the onset of precipitation, making it possible to record boundaries, as shown in Fig. 1 (A and D). In the absence of precipitation, neither inhibitory activity nor nitrogen was concentrated in the bottom cap. In contrast with precipitates obtained chemically,<sup>1</sup> these precipitates dissolved readily in dilute phosphate buffer. Indeed, resolution occurred in the cell if the current was discontinued shortly after the onset of precipitation.

During the initial period of precipitation in thick EW, the precipitate moved in the cell like a protein component too dilute to register as a distinct peak of refractive index gradient but visible because of its light-scattering capacity. The diffuse edges of this material were visible, one in each limb, moving a little slower than the ovalbumin peaks. The peaks located in the region of precipitation were initially obscured, but reappeared as the precipitate settled, and the final diagram did not differ significantly from diagrams of materials in which no precipitation occurred. With purified inhibitor, the precipitate moved well ahead of the slow component.<sup>1</sup>

*Discussion.* While the mechanism of precipitation during electrophoresis is not yet clear, the electric field, pH, and inhibitor concentration may be recognized as contributing factors. Moreover, the blending experiments indicate that the physical properties of the

# 17149. Electrical Precipitation of Egg-White Inhibitor of Influenza Virus Hemagglutination.\*

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During electrophoresis of solutions of crude egg-white or semi-purified egg-white inhibitor<sup>1</sup> of virus hemagglutination, there frequently develops a voluminous, gelatinous precipitate which contains considerable inhibitory activity. This phenomenon and its possible significance are discussed in the present report.

*Materials and methods.* Egg-white (EW) was collected from 1-day-old hen's eggs. The

thick white was obtained apart from outer and inner thin,<sup>2</sup> diluted 4-fold, and homogenized *lightly* in a Waring blender. Semi-purified inhibitor was prepared from whole or thick EW by precipitation with 7 volumes of 0.1 M  $\text{KH}_2\text{PO}_4$  and extraction of the precipitate, after washing, with 0.06 M phosphate buffer of pH 7.2<sup>1</sup>. Electrophoresis was carried out in the Tiselius cell with dialyzed solutions in veronal buffer at pH 8.6 or phos-

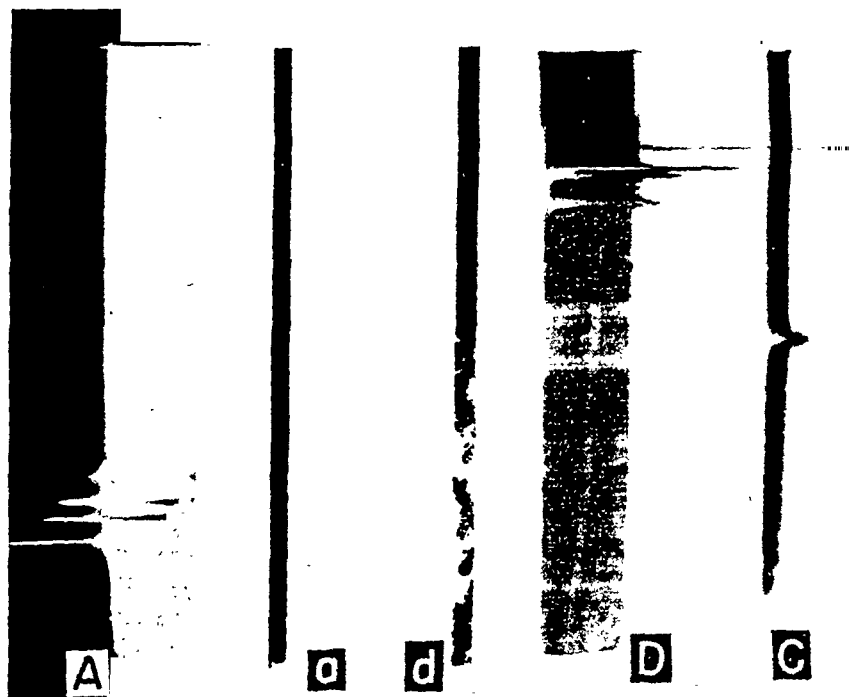


FIG. 1.

Photograph of ascending (a) and descending (d) limbs of Tiselius cell after 3 hr showing precipitate in (d). Diagrams of boundaries and the opacity due to precipitate in ascending (A) and descending (D) limbs after 22 min. The diagram of the slowest component after 215 min. is shown in C.

\* This investigation was supported by a research grant from the National Cancer Institute, U. S. Public Health Service; by a grant to Duke University from Lederle Laboratories, Inc., Pearl River, N. Y., and by the Dorothy Beard Research Fund.

<sup>1</sup> Lanni, F., Sharp, D. G., Eckert, E. A., Dillon, E. S., Beard, D., and Beard, J. W., *J. Biol. Chem.*, 1949, in press.

<sup>2</sup> Hughes, J. S., and Scott, H. M., *J. Poultry Sci.*, 1936, 15, 349.

accordance with the experience of most previous investigators, most of our attempts ended in failure. However, in studies initiated before the work of Haig<sup>4,5</sup> appeared in print, we too obtained results indicating that canine distemper virus may be successfully passed for a number of generations on the chorio-allantoic membrane of developing chick embryos. The object of this paper is to present our experimental data.

*Material and methods.* The distemper strain was the stock virus used by the Lederle Laboratories\* for the production of canine distemper vaccine and immune serum. This strain of virus, hereafter referred to as the *Lederle* strain, was isolated from a sick dog in these laboratories in 1941 and has been maintained since then by irregular alternate passages through ferrets and dogs. When inoculated into ferrets, this strain produces the usual symptoms and lesions characteristic of distemper: pyrexia, general erythema with rash around the mouth, which usually becomes pustular; erythema and edema of the anus; inflammation of the nasal passages and mucous membranes of the eyes, which is shown first by a watery discharge that later becomes purulent, causing edema and glueing of the eyelids. All inoculated ferrets show these symptoms 6 to 10 days following inoculation and usually die 2 to 3 days later.

The ferrets used in the experiments were usually between 3 and 10 months old. They were bred on an upstate New York farm and when brought to the laboratories were kept in strict isolation at an adequate distance from the infected area. They were cared for by attendants who never came in contact with dogs or the infected ferrets.

Seven-day-old developing chick embryos were used throughout. Inoculations were made either onto the chorio-allantoic (C-A) membrane or into the yolk sac. The inoculated eggs were incubated either at 35° or 38°C for 4 to 5 days at which time the chorio-allantoic membranes and/or yolk sacs, em-

bryos and allantoic fluids were harvested under aseptic conditions. All suspensions were made in beef heart infusion broth free of dextrose.

*Experimental.* Of the various routes and series of inoculations made, the series that was followed with best success is described in detail: A 20% suspension of infected ferret spleen was centrifuged at 2,000 r.p.m. for 10 minutes. To the supernate penicillin and streptomycin was added to give 500 units and 100 µg of each respectively per ml and after standing for one hour at room temperature, 12 eggs were inoculated with 0.2 ml each on the C-A membranes and then incubated at 35°C. One embryo was dead on the first day and was discarded. The remaining 11 embryos were still alive at the end of the 5th day. The C-A membranes appeared normal. They were harvested and a 20% suspension of their pooled tissue, after treatment with penicillin and streptomycin as described above, was inoculated on the C-A membranes of a second series of 15 eggs. A total of 4 serial passages in eggs was thus made. C-A membranes of the 4th passage were pooled and 0.5 ml of a 20% suspension of the pool was injected subcutaneously into each of 2 ferrets. Both ferrets developed symptoms typical of distemper 9 and 10 days later, respectively, and were found dead on the 12th day.

A 20% spleen suspension of one of the ferrets was inoculated into 10 developing chick embryos by the yolk-sac route. Four days later the yolk sacs of 8 surviving embryos were harvested and a portion of the pooled tissue suspension subinoculated onto the C-A membranes of 10 eggs. Two more serial passages were made on the C-A membranes and then pooled membranes of the last passage were made up to a 20% suspension and injected subcutaneously in 0.5 ml amounts into each of 2 ferrets. Both ferrets showed symptoms indicative of distemper on the 7th day and were found dead on the 9th day. A suspension of their pooled spleens was prepared and a portion inoculated onto the C-A membranes of 14 eggs. These eggs constituted the first passage of our current strain in eggs which now has been carried for 74 successive serial transfers on the C-A membranes.

\* The authors are indebted to Mr. H. M. Kroll, Animal Industry Section, Lederle Laboratories, for his cooperation in the initial stages of the work.

inhibitor in solution are important for the phenomenon and that it is possible to modify some of these properties without appreciably affecting biological activity. Solutions of purified inhibitor have a high viscosity, suggesting the presence of a highly asymmetric component.<sup>1,3</sup> Although it has not been possible to observe birefringence during electrophoresis of these solutions, it is possible that predisposition to aggregation results from orientation of asymmetric particles in the electric field, as in the Kerr effect.<sup>4</sup> The application of the electrical precipitation phenomenon

to the final purification of the EW inhibitor is being explored.

**Summary.** Precipitation occurs in solutions of egg-white or purified egg-white inhibitor of virus hemagglutination submitted to an electric field. The precipitate includes a great part of the inhibitor of the preparations in a state substantially purer than that of the starting material. This result suggests application of the phenomenon to the final purification of the inhibitor.

<sup>4</sup> Kerr, J., *Phil. Mag. and J. Sci.*, 4th Ser., 1875, 56, 446.

<sup>3</sup> Eckert, E. A., Lanni, F., Beard, D., and Beard, J. W., *Science*, 1949, 109, 463.

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## 17150. Propagation of Canine Distemper Virus on the Chorio-Allantoic Membrane of Embryonated Hen Eggs.

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Several attempts have been made by various investigators to cultivate canine distemper virus in the tissues of developing chick embryos. Most of these attempts were not too successful. Thus, Mitscherlich<sup>1</sup> found the virus to survive for 6 days after inoculation onto the chorio-allantoic membrane, but not to be active after subinoculation to other eggs. Plummer<sup>2</sup> in two attempts maintained the virus for 5 and 6 passages respectively on the chorio-allantois but failed to carry it further. Beveridge and Burnet<sup>3</sup> obtained no evidence that canine distemper virus multiplied in chick embryo tissues although various routes of inoculation were tried.

However, Haig<sup>4</sup> reported the successful

cultivation of Green's distemperoid virus through 30 serial passages on the chorio-allantoic membrane of chick embryos, and later reported<sup>5</sup> that the virus had been maintained on the chorio-allantoic membrane for more than 90 serial passages over a period of 15 months. During this time the egg-adapted virus was found to have become partially attenuated for ferrets and to have lost some of its highly contagious characteristics for this species. A temperature of 37°C seemed to be the most suitable for incubation of the inoculated eggs since both higher virus titers and more pronounced macroscopic lesions on the chorio-allantoic membranes were attained. Attempts to maintain the virus by serial passage by the yolk-sac, allantoic cavity or amniotic cavity routes of inoculation ended in failure.

For some time attempts have been made in this laboratory to adapt canine distemper virus to some host other than dogs or ferrets. In

<sup>1</sup> Mitscherlich, E., *Dtsch. tierarztl. Wschr.*, 1938, 46, 497.

<sup>2</sup> Plummer, P. J. G., *Canad. J. Comp. Med.*, 1939, 3, 96.

<sup>3</sup> Beveridge, W. I. B., and Burnet, F. M., *Med. Res. Council, Sp. Report Series*, No. 256, 1946, p. 76.

<sup>4</sup> Haig, D. A., *Onderstepoort J. Vet. Sci. and Animal Industry*, 1948, 23, 149.

<sup>5</sup> Haig, D. A., *J. South Afr. Vet. Med. Assn.*, 1948, 19, 73.

accordance with the experience of most previous investigators, most of our attempts ended in failure. However, in studies initiated before the work of Haig<sup>1,2</sup> appeared in print, we too obtained results indicating that canine distemper virus may be successfully passed for a number of generations on the chorio-allantoic membrane of developing chick embryos. The object of this paper is to present our experimental data.

**Material and methods.** The distemper strain was the stock virus used by the Lederle Laboratories\* for the production of canine distemper vaccine and immune serum. This strain of virus, hereafter referred to as the *Lederle* strain, was isolated from a sick dog in these laboratories in 1941 and has been maintained since then by irregular alternate passages through ferrets and dogs. When inoculated into ferrets, this strain produces the usual symptoms and lesions characteristic of distemper: pyrexia, general erythema with rash around the mouth, which usually becomes pustular; erythema and edema of the anus; inflammation of the nasal passages and mucous membranes of the eyes, which is shown first by a watery discharge that later becomes purulent, causing edema and glueing of the eyelids. All inoculated ferrets show these symptoms 6 to 10 days following inoculation and usually die 2 to 3 days later.

The ferrets used in the experiments were usually between 3 and 10 months old. They were bred on an upstate New York farm and when brought to the laboratories were kept in strict isolation at an adequate distance from the infected area. They were cared for by attendants who never came in contact with dogs or the infected ferrets.

Seven-day-old developing chick embryos were used throughout. Inoculations were made either onto the chorio-allantoic (C-A) membrane or into the yolk sac. The inoculated eggs were incubated either at 35° or 38°C for 4 to 5 days at which time the chorio-allantoic membranes and/or yolk sacs, em-

bryos and allantoic fluids were harvested under aseptic conditions. All suspensions were made in beef heart infusion broth free of dextrose.

**Experimental.** Of the various routes and series of inoculations made, the series that was followed with best success is described in detail: A 20% suspension of infected ferret spleen was centrifuged at 2,000 r.p.m. for 10 minutes. To the supernate penicillin and streptomycin was added to give 500 units and 100 µg of each respectively per ml and after standing for one hour at room temperature, 15 eggs were inoculated with 0.2 ml each on the C-A membranes and then incubated at 35°C. One embryo was dead on the first day and was discarded. The remaining 14 embryos were still alive at the end of the 5th day. The C-A membranes appeared normal. They were harvested and a 20% suspension of their pooled tissue, after treatment with penicillin and streptomycin as described above, was inoculated on the C-A membranes of a second series of 15 eggs. A total of 4 serial passages in eggs was thus made. C-A membranes of the 4th passage were pooled and 0.5 ml of a 20% suspension of the pool was injected subcutaneously into each of 2 ferrets. Both ferrets developed symptoms typical of distemper 9 and 10 days later, respectively, and were found dead on the 12th day.

A 20% spleen suspension of one of the ferrets was inoculated into 10 developing chick embryos by the yolk-sac route. Four days later the yolk sacs of 8 surviving embryos were harvested and a portion of the pooled tissue suspension subinoculated onto the C-A membranes of 10 eggs. Two more serial passages were made on the C-A membranes and then pooled membranes of the last passage were made up to a 20% suspension and injected subcutaneously in 0.5 ml amounts into each of 2 ferrets. Both ferrets showed symptoms indicative of distemper on the 7th day and were found dead on the 9th day. A suspension of their pooled spleens was prepared and a portion inoculated onto the C-A membranes of 14 eggs. These eggs constituted the first passage of our current strain in eggs which now has been carried for 74 successive serial transfers on the C-A membranes.

\* The authors are indebted to Mr. H. M. Kroll, Animal Industry Section, Lederle Laboratories, for his cooperation in the initial stages of the work.



inhibitor in solution are important for the phenomenon and that it is possible to modify some of these properties without appreciably affecting biological activity. Solutions of purified inhibitor have a high viscosity, suggesting the presence of a highly asymmetric component.<sup>1,3</sup> Although it has not been possible to observe birefringence during electrophoresis of these solutions, it is possible that predisposition to aggregation results from orientation of asymmetric particles in the electric field, as in the Kerr effect.<sup>4</sup> The application of the electrical precipitation phenomenon

to the final purification of the EW inhibitor is being explored.

**Summary.** Precipitation occurs in solutions of egg-white or purified egg-white inhibitor of virus hemagglutination submitted to an electric field. The precipitate includes a great part of the inhibitor of the preparations in a state substantially purer than that of the starting material. This result suggests application of the phenomenon to the final purification of the inhibitor.

<sup>4</sup> Kerr, J., *Phil. Mag. and J. Sci.*, 4th Ser., 1875, **56**, 446.

<sup>3</sup> Eckert, E. A., Lanni, F., Beard, D., and Beard, J. W., *Science*, 1949, **109**, 463.

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# 17150. Propagation of Canine Distemper Virus on the Chorio-Allantoic Membrane of Embryonated Hen Eggs.

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Several attempts have been made by various investigators to cultivate canine distemper virus in the tissues of developing chick embryos. Most of these attempts were not too successful. Thus, Mitscherlich<sup>1</sup> found the virus to survive for 6 days after inoculation onto the chorio-allantoic membrane, but not to be active after subinoculation to other eggs. Plummer<sup>2</sup> in two attempts maintained the virus for 5 and 6 passages respectively on the chorio-allantois but failed to carry it further. Beveridge and Burnet<sup>3</sup> obtained no evidence that canine distemper virus multiplied in chick embryo tissues although various routes of inoculation were tried.

However, Haig<sup>4</sup> reported the successful

cultivation of Green's distemperoid virus through 30 serial passages on the chorio-allantoic membrane of chick embryos, and later reported<sup>5</sup> that the virus had been maintained on the chorio-allantoic membrane for more than 90 serial passages over a period of 15 months. During this time the egg-adapted virus was found to have become partially attenuated for ferrets and to have lost some of its highly contagious characteristics for this species. A temperature of 37°C seemed to be the most suitable for incubation of the inoculated eggs since both higher virus titers and more pronounced macroscopic lesions on the chorio-allantoic membranes were attained. Attempts to maintain the virus by serial passage by the yolk-sac, allantoic cavity or amniotic cavity routes of inoculation ended in failure.

For some time attempts have been made in this laboratory to adapt canine distemper virus to some host other than dogs or ferrets. In

<sup>1</sup> Mitscherlich, E., *Dtsch. tierarztl. Wschr.*, 1938, **46**, 497.

<sup>2</sup> Plummer, P. J. G., *Canad. J. Comp. Med.*, 1939, **3**, 96.

<sup>3</sup> Beveridge, W. J. B., and Burnet, F. M., *Med. Res. Council, Sp. Report Series*, No. 256, 1946, p. 76.

<sup>4</sup> Haig, D. A., *Onderstepoort J. Vet. Sci. and Animal Industry*, 1948, **23**, 149.

<sup>5</sup> Haig, D. A., *J. South Afr. Vet. Med. Assn.*, 1948, **19**, 73.

lethal effect for ferrets through at least the 20th egg passage. Starting at the 26th passage level, however, the C-A membrane material was definitely of lessened virulence since 1 of 2 ferrets survived infection following a very mild illness and was found to be completely resistant on rechallenge with approximately 100 MLD of virulent ferret spleen virus 30 days later. Likewise ferrets inoculated with C-A membranes of the 28th and 32nd egg passages showed no signs of infection although they too were found completely resistant to rechallenge with approximately 100 MLD of virulent ferret spleen virus 30 days later.

*Further proof for identity of the virus.* In order to eliminate the possibility of having viruses other than that of canine distemper present, the following animals were inoculated with a 20% suspension of C-A membranes of the 48th egg passage: 6 Swiss albino mice with 0.03 ml each intracerebrally; 3 guinea pigs with 0.15 ml each intracerebrally; 3 guinea pigs with 1.0 ml each subcutaneously; 2 rabbits on the scarified corneas with 0.1 ml each, and 2 ferrets with 1.0 ml each intraperitoneally. None of the inoculated animals showed any signs of illness, therefore infection with other viruses such as lymphocytic choriomeningitis or herpes simplex was improbable. The 2 ferrets were challenged at the end of 30 days and were shown to be immune to at least 100 MLD of virulent ferret spleen distemper virus.

*Neutralization tests in eggs.* Dogs, ferrets and guinea pigs were hyperimmunized with repeated injections of ferret spleen virus. Blood samples were drawn and the sera from each species pooled. Pools of normal ferret serum and normal guinea pig serum were also prepared. The 5 serum pools were filtered separately through single Seitz EK pads and inactivated at 56°C for 30 minutes. Each serum pool was then mixed with an equal volume of a 20% suspension of C-A membranes of the 55th egg passage. As a further control a mixture of equal volumes of sterile broth and C-A membrane suspension was similarly prepared. All 6 mixtures were incubated at 37°C for 2 hours before inoculation into 6 eggs each on the C-A membranes.

All eggs were opened after 5 days' incubation at 35°C and the embryonic membranes and tissues carefully examined macroscopically. The results may be summarized by stating that those eggs inoculated with the mixtures containing normal ferret serum, normal guinea pig serum or sterile broth all showed the markedly thickened membranes and numerous opaque foci that are usually observed with the *Lederle* strain of egg-adapted canine distemper virus. In contrast those eggs inoculated with the mixtures containing immune guinea pig, ferret or dog sera showed only the typically thin, sparkling transparent appearance of normal membranes with no visible lesions whatsoever.

*Neutralization tests in ferrets.* Two ferrets were hyperimmunized by injecting them subcutaneously at 5-day intervals with 1 ml of a 20% C-A membrane suspension representing the 59th, 60th, 61st, 62nd, 63rd and 64th egg passages. The ferrets were bled by cardiac puncture one week after the last injection and their sera pooled. Three guinea pigs which were hyperimmunized in a similar manner were likewise bled and their sera pooled. In addition 2 other ferrets were submitted to the same schedule of immunization with the exception that they received an additional intraperitoneal injection of 2.0 ml of a 20% suspension of the 65th C-A membrane passage. They were also bled one week after the last injection and their sera pooled. Pools of normal ferret and of normal guinea pig sera were also prepared. The 5 serum pools were filtered separately through single Seitz EK pads and inactivated at 56°C for 30 minutes. Mixtures, containing 8 ml of the respective serum plus 2 ml of infected ferret spleen suspension (containing at least 100 MLD of virulent virus per ml) were incubated at 37°C for 2 hours and then injected intraperitoneally into 2 ferrets, each ferret receiving 5 ml of the serum-virus mixture. The results may be summarized by stating that those ferrets inoculated with the mixtures containing normal ferret or guinea pig serum showed typical symptoms of distemper starting either on the 7th or 8th day and were dead by the 11th to 13th day. In contrast the 6 ferrets inoculated with the

Our experience with the *Lederle* strain propagated on the C-A membranes has been very similar in many ways to that reported by Haig<sup>4,5</sup> for the *Onderstepoort* strain. Up through the 9th to 10th passage of the *Lederle* strain very little, if any, changes were noticed in the appearance of the C-A membranes. However, starting at that passage level some of the membranes were noted to be thickened and moist, showing fairly numerous small areas whitish-gray in appearance. These changes became more marked with increasing number of egg passages so that to date, at the 74th passage, the membranes are markedly thickened and the accompanying lesions quite numerous, heavy and opaque (see Fig. 1).

Appropriate bacteriological tests carried out routinely with both aerobic and anaerobic media have consistently shown the infected tissues to be free of any detectable bacterial contaminants.

*Subinoculation of the egg-adapted virus into ferrets.* At intervals membranes of a certain passage level were pooled, homogenized to a uniform suspension and inoculated into ferrets for testing of virulence. The data

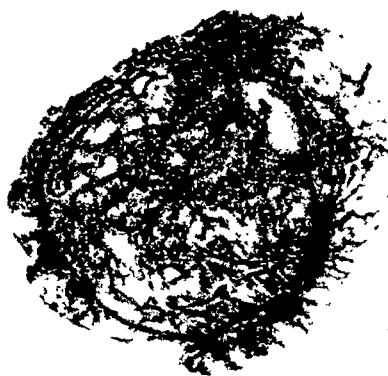


FIG. 1.

Distemper infected chorio-allantoic membrane.

pertaining to the maintenance of the virus in eggs and subinoculation into ferrets are summarized in Fig. 2.

In brief, it was found that canine distemper virus may be maintained by serial passage on the C-A membranes of developing chick embryos and that the virus still retains its

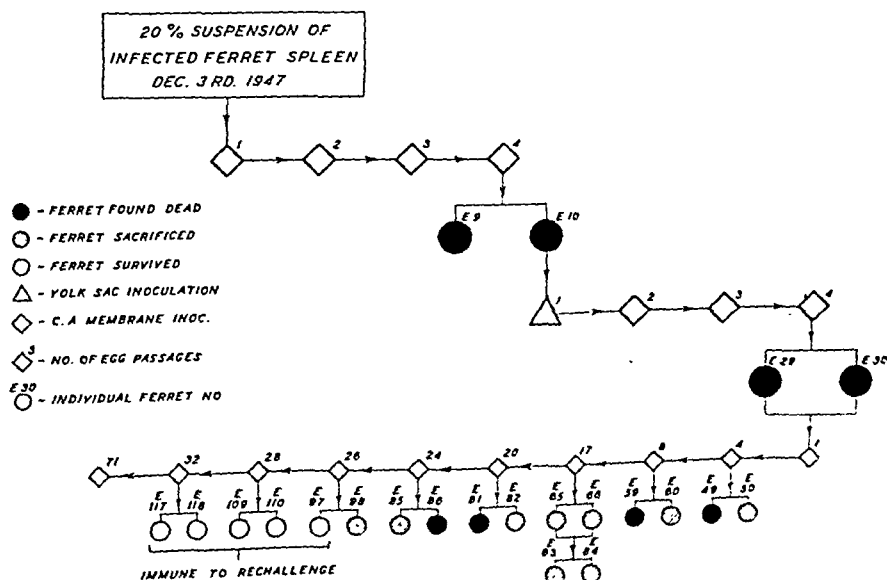


FIG. 2.

Ferret and egg passage series of distemper virus.

blood count, sedimentation rate, bleeding and coagulation time, serum protein, alkaline phosphatase, cephalin flocculation, thymol turbidity, NPN, and serum chymotrypsin and rennin inhibitors.<sup>3</sup> When feasible, patients were followed roentgenographically as well.

The diagnosis in each instance had been proved by biopsy. There were 15 cases in the series: bronchogenic carcinoma, 3; carcinoma of stomach, 2; embryonal carcinoma of testicle, 2; and one each of recto-sigmoid and uterine carcinoma, synovium, lymphosarcoma, Hodgkin's disease, acute and subacute lymphatic leukemias, and chronic myelogenous leukemia.

Crystalline chymotrypsin in saline was given in daily intramuscular doses ranging from 4 to 60 mg for periods of 21 to 135 days, with an average of 64 days. The usual dose was the recommended one of 30 mg. Three patients could not tolerate more than 4 to 6 mg without intense pain in the most involved organ (liver, stomach and spleen) accompanied by chills and fever 12 to 24 hours after injection. Six cases developed pronounced allergy to the drug, forcing reduction in dose or discontinuation of treatment. Two had generalized urticarial reactions, 2 exhibited

anaphylactic shock, and 2 had extreme local erythema and edema at the site of injection. Two patients died in congestive failure about a week after onset of precordial pain, ankle edema and dyspnea. Electrocardiograms were normal before death and autopsy failed to reveal significant cardiac pathology. Whether or not these deaths were related to treatment is debatable.

The sedimentation rate was doubled during chymotrypsin therapy. There were no other consistent laboratory findings. The serum level of chymotrypsin inhibitor was not altered detectably, which is understandable in view of the fact that there is sufficient inhibitor in the circulation alone in many such patients to inactivate 25 to 50 g of pure chymotrypsin.

In no case was the clinical course of the patient influenced by chymotrypsin therapy. Nine patients have died and a study of autopsy material has not revealed any unusual degenerative changes in the neoplasm.

*Summary.* Treatment with crystalline chymotrypsin did not influence the course of neoplastic disease in experimental animals or in fifteen selected cancer patients.

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### 17153. Action of Chloromycetin on Salmonella.

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As yet there exists no effective remedy to prevent or cure *Salmonella* infections in man and animals. Any new antibiotic with selective activity against gram negative organisms, therefore, is worth a trial against *Salmonella* infections. Chloromycetin, the antibiotic produced by *Streptomyces Venezuelae*<sup>1</sup> possesses these qualities in addition to its marked effect against *Rickettsia*. Clinical experiments indicate some success in the treatment of hu-

man typhoid fever (a *Salmonellosis*).<sup>2</sup>

*In vitro* tests with a number of gram negative bacilli were performed by Smith *et al.*,<sup>3</sup> and revealed remarkable sensitivity, usually in a range below 1  $\mu$ g of the drug. These results were obtained by a special turbidity assay method developed by Joslyn and Gal-

<sup>2</sup> Woodward, T. E., Smadel, J. E., Ley, H. L., Jr., Green, R., and Mankikar, D. S., *Ann. Int. Med.*, 1948, 29, 131.

<sup>3</sup> Smith, R. M., Joslyn, D. A., Grubitz, O. M., McLean, I. W., Jr., Penner, M. A., and Ehrlich, J., *J. Bact.*, 1948, 55, 425.

<sup>1</sup> Ehrlich, J., Bartz, Q. R., Smith, R. M., Joslyn, D. A., and Burkholder, P. R., *Science*, 1947, 100, 417.

of marked diaphoresis, weakness, loss of consciousness, rapid thready pulse, faint heart sounds, and peripheral vascular collapse were encountered in 3 patients, (Cases 8, 9 and 10) within 5 minutes following the 40th, 53rd and 29th doses, respectively, of the enzyme. Prompt use of artificial respiration, epinephrine intramuscularly, oxygen inhalations and intravenous saline probably prevented fatal termination of these anaphylactoid reactions in patients apparently sensitized by previous injections of the protein enzyme. Another patient (Case 6) had a similar but

less severe reaction with spontaneous recovery after the 22nd dose.

Further trial of chymotrypsin was discontinued, not only because it had no effect whatsoever in 10 patients with advanced malignant neoplasms, but because repeated administrations of the protein enzyme are of definite serious hazard.

*Summary.* Parenteral injections of chymotrypsin had no effect on the course of 10 cases of neoplastic disease in man.

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### 17152. Ineffectiveness of Chymotrypsin Therapy in Malignancy.\*

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An elaborate theory has been proposed by Krebs and co-workers<sup>1,2</sup> which assumes that the cancer cell and the trophoblast cell are fundamentally alike, and that both are destroyed selectively by the pancreatic enzyme chymotrypsin. On the basis of this reasoning, but with few supporting facts, chymotrypsin has been suggested as the palliative of choice in neoplastic disease. This report summarizes the negative results of chymotrypsin therapy in experimental and clinical cancer.

*Experimental cancer.* The toxic dose of crystalline chymotrypsin<sup>†</sup> in mice subjected to repeated daily intraperitoneal injection was established at 40 to 80 mg per kilo of body weight. The dose used in treatment was 2.0

mg per kilo, which is 5 times the recommended dose for humans. Forty mice with subcutaneously transplanted sarcoma 180 and 40 without tumor were employed in the experiment. Half of each group received chymotrypsin intraperitoneally 7 days a week for 14 days, the remainder serving as controls. Weight gains were uniform in all 4 groups of animals. Treatment was begun when tumors were measurable, on the fifth day after transplantation, and tumor size was recorded on alternate days thereafter. The average increase in tumor area at the conclusion of the experiment was 3.8 times in the treated animals and 3.7 times in the controls. No change in chymotrypsin inhibitor concentration of the blood as determined by the method of West and Hilliard<sup>3</sup> resulted from the treatment in either normal or tumor-bearing animals.

*Clinical Cancer.* All patients selected for chymotrypsin treatment had received the maximum benefit from accepted forms of therapy but still had active disease. Their clinical course was followed by an impartial group of observers, Drs. G. M. Leiby, J. A. Weinberg, F. Isaac, and F. W. Wilkins of this hospital. Weekly laboratory determinations included,

\* Aided by a grant from the California Institute for Cancer Research. Published with permission of the Chief Medical Director, Department of Medicine and Surgery, Veterans Administration, who assumes no responsibility for the opinions expressed or conclusions drawn by the authors.

<sup>1</sup> Gurehot, C., Krebs, E. T., Jr., and Krebs, E. T., *Surg., Gynec. and Obst.*, 1947, **84**, 301.

<sup>2</sup> Krebs, E. T., Krebs, E. T., Jr., and Gurehot, C., *M. Rec.*, 1947, **160**, 479.

<sup>†</sup> Crystalline Chymotrypsin was supplied by Spicer-Gerhart Co., Pasadena, Calif., and Worthington Biochemical Laboratory, Freehold, N. J.

<sup>3</sup> West, P. M., and Hilliard, Jessamine, in press.

TABLE I.  
Oral Infection of Mice with 0.1 cc of Broth Culture of *S. typhi murium* 1908 and Treatment with Chloromycetin.

Treatment	No. of mice	Surviving after, days										
		1	5	6	7	8	9	10	11	12	14	21
None (controls)	20	20	16	12	9	6	3	1	0			
Oral	20	10	10	10	10	8*	7	7	5	5	2	1
Parenteral	10	10	10	10	10	9*	9	9	8	7	6	1

\* Last day of treatment.

TABLE II.  
Parenteral Infection of Mice with 0.1 cc of Broth Culture of *S. typhi murium* 1908 (1:60) and Treatment with Chloromycetin.

		Surviving after, days										
Treatment	No. of mice	1	5	6	7	8	9	10	11	12	14	21
None (controls)	20	20	7	5	4	0						
Oral	10	10	10	10	10	8*	7	7	5	5	2	1
Parenteral	20	20	20	18	15*	11	10	9	6	5	3	3

\* Last day of treatment.

days. Total injected chloromycetin was 35 mg or 1750 mg per kg. Table I gives the results.

Treatment began immediately after infection and terminated on the eighth day. On the 11th day all controls were dead, while 5 and 8, respectively, of the treated animals were still alive. At the end of the experiment, however, (21st day) only one of each series of treated animals survived. Thus, oral infection with *S. typhi murium* could not be checked by oral or subcutaneous treatment with large doses of chloromycetin. In Table II similar experiments were performed after parenteral infection of the mice (.1 cc of an 18 hour broth culture of *S. typhi murium* 1908, diluted 1:60). The parenteral treatment with chloromycetin was performed for 7 days with a total of 25 mg or 1250 mg per kg; the oral treatment (8 days) with a total of 24 mg or 1200 mg per kg.

The results are almost identical with those of the first test. When all untreated animals had succumbed (7th day), a considerable number of treated mice were still alive. On withdrawal of the drug, however,

and in the course of the next 2 weeks, most of the treated animals died. Only a few survived at the end of the experiment (21st day). *S. typhi murium* was recovered from the controls and the treated dead mice, often in pure culture. It was found in heart blood and intestines in both series. The chloromycetin sensitivity of the recovered strains had not changed. The original culture, as well as the strains isolated from the animals, still showed sensitivity to 2  $\mu$ g.

*Summary.* Chloromycetin inhibits the growth of *Salmonella* organisms *in vitro* remarkably, but is not able even in large amounts to control *Salmonella* infections in mice. Neither the oral nor the subcutaneous form of treatment prevented the fatal outcome of the disease in most animals, although it prolonged the survival time. Its action in the animals was bacteriostatic and not bactericidal. Due to its absorption conditions, chloromycetin exerts no influence on the intestinal flora, when given orally.

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braith.<sup>4</sup> A strain of *Sh. sonnei* No. 04628 was used as the test strain, and inhibited by .2  $\mu$ g in this set-up. In our experiments\* the same test strain was employed in a simplified serial dilution method. Sensitivity was measured by the smallest amount of chloromycetin that completely suppressed growth after 24 hours. With this method the test strain was regularly sensitive to 1  $\mu$ g.

Sixty-eight gram negative strains were examined; they included *B. coli* and other coliform organisms, *aerobacter aerogenes*, *B. proteus*, *Pseudomonas aeruginosa*, *Shigella* types, 2 strains of *S. typhosa* and 21 other *Salmonella* types. Most strains of *Pseudomonas aeruginosa* and some coliform organisms were remarkably resistant to chloromycetin and required concentrations from 20-100  $\mu$ g per ml for inhibition. Of the 23 *Salmonella* types, 19, including *S. typhosa*, were sensitive to 2  $\mu$ g, four to 4  $\mu$ g, none higher. No relation existed between streptomycin resistance and chloromycetin resistance; no absolute resistance to chloromycetin was encountered in any strain.

For animal experiments (oral and parenteral treatment) solutions of chloromycetin in 20% propylene glycol were used; emulsions in acacia, as recommended, were not practicable because of the small amount of the material to be ingested.

Crystallized chloromycetin is not readily soluble in water; suitable solutions however, can be produced by use of 20% propylene glycol. Final concentrations of 10 mg per ml were obtained by heating for 10-20 minutes in a water bath at 56°. At room temperature needle-shaped crystals precipitated. They disappeared on repeated immersion into the water bath at 56°. The antibiotic activity of these solutions was not impaired by repeated exposure to 56°. Mice, 20 g of weight were fed with chloromycetin solution by introduction through a smoothly blunt 18 gauge needle attached to a tuberculin-syringe. For 4 consecutive days 2000  $\mu$ g

(contained in .2 ml) were given twice daily, at 9 A.M. and 4 P.M. The total dose amounted to 16 mg or 800 mg per kg mouse. No toxic effects were observed.

The intestinal flora was checked by stool cultures taken before the start of the experiment and after the 5th and 7th feeding. No growth difference was found; number and size of colonies (mostly *B. coli*, *B. proteus*, enterococci), remained almost unchanged during the course of the tests. In this respect the action of chloromycetin is different from that of streptomycin. Feeding of the latter drug results in a marked decrease of fecal organisms, even bringing about complete inhibition of growth.<sup>5,6</sup> Streptomycin remains practically unabsorbed in the intestinal tract, and is not destroyed in the intestines. Since chloromycetin is readily absorbed by the oral route,<sup>7</sup> no active material remains in the intestines for any length of time. The fecal flora, therefore, is not affected.

*Salmonella infections.* Virulent organisms used for infection of mice by the oral or parenteral route, as a rule, give rise to generalized septicemia with fatal outcome. The bacilli transgress the intestinal wall from both sides, from the intestinal into the general circulation as well as from blood and lymph system into the intestinal lumen. The failure, therefore, of chloromycetin given by mouth to influence the bacteria inside the intestinal tract does not preclude its possible activity against those *Salmonella* organisms that have entered the body system from there.

In the first experiment 40 mice of about 20 g body weight received orally .1 cc of an 18 hour broth culture of *S. typhi murium* 1908 known for its constant virulence. Twenty animals served as controls, 10 mice underwent oral treatment: 2000  $\mu$ g twice a day for 4 days, once a day for 4 more days; total ingested chloromycetin was 24 mg or 1200 mg per kg. A second batch of 10 mice was injected subcutaneously with 2500  $\mu$ g twice a day for 6 days and once a day for 2 more

\* Joslyn, D. A., and Galbraith, M., *J. Bact.*, 1947, 54, 26.

\* Crystallized chloromycetin was supplied through the courtesy of Parke Davis and Co.

<sup>5</sup> Seligmann, E., and Wassermann, M., *J. Bact.*, 1946, 53, 127.

<sup>6</sup> Seligmann, E., Barash, L., and Coblan, S. Q., *J. Ped.*, 1947, 20, 182.



FIG. 1.

Small-nerve fiber stimulation in ventral root, recording from dorsal rootlet. Single fiber afferent discharges from soleus at varying muscle tensions. Stimulus intervals are 9 msec.

A, B, and C—Muscle tension 40 g. In A and B 2 and 3 stimuli set up 2 sensory spindle discharges. C, 6 stimuli cause a train of responses.

D and E—Tension 20 g. D, 6 stimuli as in C now set up fewer discharges. E, 10 stimuli restore longer train of discharges.

F and G—Zero tension. 6 and 10 stimuli are practically ineffective. Occasional resting discharges occurred at 40 and 20 g muscle tension.

number of stimuli but shows a mechanism of facilitation. Lowering the muscle tension renders the spindle less sensitive to small-nerve excitation, but at intermediate tensions an increase in number of stimuli can compensate for such an effect (C + E). However, at zero muscle tension even prolonged stimuli remain practically ineffective. The spindle of Fig. 1 gave an  $A_1$  type response as described by Matthews. Its discharge showed a high threshold to stretch.

The facilitation mechanism of spindle discharge and other data suggest that the small-nerve fibers in the cat cause local contractions in intrafusal muscle fibers. This would be analogous to the graded contractile responses in frog striated muscles, innervated by the small-nerve motor system.

Besides the small-nerve innervation de-

scribed here, many spindle intrafusal fibers are supplied by motor nerves of larger diameter as originally found by Matthews (cf also<sup>10</sup>). The latter cause a discharge pattern which is in contrast to the one illustrated in Fig. 1 in that a single nerve impulse sets up a burst of sensory discharges.

**Summary.** The lumbosacral ventral root outflow of the cat contains 20-25 p.c. small diameter nerve fibers. These cause no detectable shortening of muscles and no propagated muscle impulses. Stimulation of such fibers sets up discharges from muscle spindle receptors. The discharge is dependent upon the number and frequency of stimuli and on adjustment of muscle stretch.

<sup>10</sup> Barker, D., *Quart. J. Microscop. Sci.*, 1948, 89, 143.



## 17154. Small-nerve Fibers in Mammalian Ventral Roots.\*

STEPHEN W. KUFFLER AND CARLTON C. HUNT.† (Introduced by Philip Bard.)

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The function of the frogs small-nerve motor system has been studied in detail during the last few years.<sup>1-4</sup> Slowly conducting high threshold nerve fibers of around 5  $\mu$  in diameter emerge through the ventral roots and innervate numerous muscles in the body. On stimulation they cause local graded contractions around the neuromuscular junction, depending on the frequency of stimulation. The frogs small-nerve system also shows a distinct reflex pattern.

In the cat about 25 per cent of the nerve fibers in the ventral roots of the lumbosacral region are medullated fibers of small diameter with a distribution peak of 4-6  $\mu$ .<sup>5</sup> Their physiological properties have been studied by several investigators.<sup>1,6,7</sup> In particular Leksell<sup>8</sup> concluded that the effect of the small-nerve fibers on the contractile mechanism was small or absent and that the fibers influenced the afferent discharge from muscle proprioceptors.

A systematic investigation was made of the function of ventral root nerve fibers to the soleus and tenuissimus. By subdividing ventral roots into fine filaments individual nerve fibers innervating different muscles were ob-

tained. The nerve fibers were then stimulated singly or together at varying frequencies and their effect was determined on the contractile and sensory mechanism of the muscles. Nerve fibers conducting at speeds faster than 50 m/sec caused the well known motor unit response, *i.e.* twitches and propagated muscle impulses. Nerve fibers conducting between 16-50 m/sec. caused no detectable muscle shortening and no propagated muscle impulses. Calculations of diameters from conduction velocities in the small-nerve group showed a distribution in agreement with histological data (3-8  $\mu$ , cf<sup>9</sup>).

The sensory discharges of muscles could be detected by recording from the surface of the tenuissimus, from nerve branches emerging from muscles, or preferably by isolating filaments of dorsal roots, containing a single nerve fiber from the muscle under study. The latter method gave simple and clear-cut indication of the sensory discharge regulation in muscle spindles, since a single efferent fiber to a muscle spindle could be stimulated and its effect recorded in a single sensory fiber. Different discharge patterns discovered by Matthews<sup>9</sup> were easily detected and his findings of A<sub>1</sub>, A<sub>2</sub> and B discharge types were readily confirmed in numerous observations.

Stimulation of the small ventral root fibers, when effective, increased the afferent discharge from muscle spindles, dependent upon the tension of the muscle and the duration and frequency of stimulation. The increase could be elicited from spindle receptors which showed a steady rate of discharge or from receptors which showed no resting discharge at a given initial tension. The principal feature of the small-nerve mechanism is illustrated in Fig. 1, which shows that the sensory discharge can be finely regulated by adjustment of muscle tension and by motor excitation of the spindle intrafusal fibers. In A, B, and C it is shown that the discharge is not directly proportional to the

\* Supported by a grant from the National Foundation for Infantile Paralysis, Inc., and by the Rockefeller Foundation.

† Senior Fellow of the National Research Council.

<sup>1</sup> Kuffler, S. W., and Gerard, R. W., *J. Neurophysiol.*, 1947, **10**, 383.

<sup>2</sup> Kuffler, S. W., Laporte, Y., and Ransmeier, R. E., *J. Neurophysiol.*, 1947, **10**, 395.

<sup>3</sup> Tasaki, I., and Mizutani, K., *Jap. J. med. Sci.*, 1944, **10**, 237.

<sup>4</sup> Tasaki, I., and Tsukagoshi, M., *Jap. J. med. Sci.*, 1944, **10**, 245.

<sup>5</sup> Eccles, J. C., and Sherrington, C. S., *Proc. Roy. Soc.*, 1930, **106B**, 326.

<sup>6</sup> Hügghquist, G., *Acta Med. Scand.*, 1940, **104**, S.

<sup>7</sup> O'Leary, J., Heinbecker, P., and Bishop, G. H., *Am. J. Physiol.*, 1934, **110**, 636.

<sup>8</sup> Leksell, L., *Acta physiol. Scand.*, 1945, **10**, suppl. 31, 84 pp.

<sup>9</sup> Matthews, B. H. C., *J. Physiol.*, 1933, **78**, 1.

TABLE I.  
Staphylocoagulability of Various Plasmas, at 37°C and 20°C. Clotting-times with Prostaphylocoagulase; A. with, B. without, Added Cofactor.

Plasma	A. Prostaph. + cofactor		B. Prostaph. + buffer	
	37°C	20°C	37°C	20°C
Rabbit	19 min.	22 min.	18 min.	17 min.
Human	36 min.	54 min.	36 min.	54 min.
Chicken	No clot*	No clot*	No clot*	No clot*
Rat	" "	" "	" "	" "
Guinea pig	240 min.†	324 min.	No clot‡	323 min.
Dog	73 min.	29 min.	180 min.†	28 min.
Horse	100 min.	235 min.	227 min.	228 min.
Bovine	150 min.	660 min.	660 min.	660 min.

\* Thrombin added after 24 hr gave solid clot; no fibrinogenolysis.

† Clot not quite solid; fibrinolysis subsequently.

‡ Thrombin added after 24 hr gave no clot; fibrinogenolysis.

II. albumin supernatant from the original globulin precipitation: similarly dialyzed and concentrated to original plasma volume; 6. cofactor (activator) of prostaphylocoagulase was human serum albumin prepared as above.

*Factors affecting staphylocoagulation in plasmas of various species.* Tests were run at 20° and 37°C, timing the coagulation of 0.5 ml plasma with A. 0.25 ml prostaphylocoagulase + 0.25 ml cofactor; B. 0.25 ml prostaphylocoagulase + 0.25 ml buffer. When no clotting was evident after 24 hours, 0.25 ml thrombin was then added to determine whether fibrinogenolysis had occurred incidental to the staphylokinase-protease phenomenon. In order to determine inhibitory effects directed against active staphylocoagulase, prostaphylocoagulase and cofactor were incubated at 37° for half an hour before adding the selected plasma. Thus, species differences in staphylocoagulation times could be evaluated, at least semiquantitatively, with respect to (1) low cofactor content; (2) presence of inhibitors (s); 3. interference by protease, or (4) several possible combinations of these factors. The initial data are summarized in Table I, and their analysis is supplemented by the special tests of Tables II and III.

These experiments indicate: 1. Staphylocoagulation times, under carefully controlled test conditions, vary according to species of plasma; 2. Several plasma factors contribute to these species variations, e.g. (A) Rabbit, dog, human, and to a lesser extent horse, guinea pig, and bovine, give staphylocoagu-

lation with the bacterial product alone (the citrate precluding any possible thrombic mechanism) and must, therefore, contain cofactor: (B) additional cofactor reduces the coagulation times of horse and bovine plasma, but not to the level of the best clotting times seen with rabbit plasma. Proteolysis is not evident in these species. Hence it is concluded that they are relatively lower in cofactor than the rabbit and also contain some inhibitor: (C) Human plasma gives the same staphylocoagulation times, with or without cofactor, and longer than those for rabbit plasma. In the absence (in these particular experiments) of proteolytic effects, the explanation must be in terms of inhibitor(s): (D) That at least part of the inhibitory effects is against active staphylocoagulase is proved by the method stated. Such inhibitor is very marked in chicken and rat plasmas which do not clot under any of the test conditions, including the addition of preincubated prostaphylocoagulase + cofactor. Since clots occur on the addi-

TABLE II.  
Inhibitory Effects of Various Plasma Dilutions; 0.25 ml Added to Mixture\*: 1 ml Fibrinogen, 0.5 ml Cofactor, 0.25 ml Prostaphylocoagulase. Clotting-times (min.) at 37°C.

Species *	Plasma dilutions				
	1:1	1:8	1:32	1:128	1:256
Chicken	2160	119	44	44	42.5
Horse	318	62	62	40	40
Guinea pig	80	80	64.5	64.5	41

\* Control (buffer instead of plasma): 40 min. staphylocoagulation time.

## 17155. Staphylocoagulation in Plasmas of Various Animal Species.\*

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Smith and Hale<sup>1</sup> showed that clotting of decalcified plasma by staphylococci takes place in two stages, namely, (1) reaction of the bacterial product (prostaphylocoagulase<sup>2</sup>) with a cofactor or activator-substance in plasma to form the active clotting agent (staphylocoagulase), which (2) then converts fibrinogen into a fibrin which, according to our own observations under the dark-field microscope, appears indistinguishable from ordinary fibrin, despite the fact, which we have indubitably confirmed, that the staphylocoagulation phenomenon can occur in the complete absence of the thrombin mechanism.<sup>2</sup> We have previously reported<sup>3</sup> on the occurrence of cofactor (Tager's<sup>4,5</sup> "coagulase reacting factor") in various blood and tissue products, finding a human serum albumin (see reagents) to be the most satisfactory source material.

In earlier literature on the great variability of the staphylocoagulation phenomenon in plasmas of differing animal species, some suggestions have been made as to lack of cofactor (activator), thermolability of activator substance or of staphylocoagulase, and possible combinations of these and other factors. For instance, it has been stated<sup>1</sup> that fowl and mouse plasmas completely lack activator sub-

stance, whereas guinea-pig plasma clots at 20°C but not at 37° owing to low concentration of activator, in consequence of which staphylocoagulase production proceeds at too slow a rate to keep pace with thermal deterioration at the higher temperature (cf. *infra*). Kaplan and Spink<sup>6</sup> give evidence for a further factor, namely, an inhibitor, demonstrable in the euglobulin fraction of human and guinea-pig plasmas. It is precipitable between 25 and 40% saturation with ammonium sulfate. Tager and Hales<sup>7</sup> also suggest that the coagulase reacting factor may be bound or masked in the plasma of certain species.

The present study is an extension of earlier reports<sup>2,3</sup> and describes experiments in which the plasmas of a number of animal species have been tested with some modified methods and fractionated with classical ammonium sulfate procedures in an attempt to explain more fully the reasons for reduction or absence of staphylocoagulation in the case of certain species.

*Reagents.* 1. The borate buffer, pH = 7.7, used as solvent and diluent throughout, was citrated to 0.4%  $\text{Na}_2\text{C}_6\text{H}_5\text{O}_7$ ,  $5\frac{1}{2}$   $\text{H}_2\text{O}$ ; 2. a 1% staphylococcal product, containing both prostaphylocoagulase and staphylokinase, was prepared as described previously;<sup>3</sup> 3. Fibrinogen was Armour's bovine plasma Fraction-I; 4. Thrombin (10 units/ml) was diluted commercial (Parke, Davis) "Thrombin Topical"; 5. The plasmas of the various species were centrifuged in citrate (0.4%) and, for some tests, fractionated with ammonium sulfate into the classical crude "globulins" and "albumin" as follows: I. 50% saturation with  $(\text{NH}_4)_2\text{SO}_4$  precipitated the globulins which, on dialysis until sulfate-free yielded (a) "euglobulin" precipitate: dissolved in citrated borate buffer to original plasma volume. (b) "pseudoglobulin" supernatant: dialyzed and concentrated *in vacuo* to original plasma volume:

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† Predoctorate Research Fellow, U. S. Public Health Service. Present address: Wayne University College of Medicine, Department of Physiology and Pharmacology, Detroit, Mich.

<sup>1</sup> Smith, W., and Hale, J. H., *Brit. J. Exp. Path.*, 1944, **25**, 101.

<sup>2</sup> Gerheim, E. B., Ferguson, J. H., and Travis, B. L., *Fed. Proc.*, 1948, **7**, 41.

<sup>3</sup> Gerheim, E. B., Ferguson, J. H., and Travis, B. L., *Proc. Soc. Exp. Biol. and Med.*, 1947, **66**, 325.

<sup>4</sup> Tager, M., *Yale J. Biol. Med.*, 1948, **20**, 369.

<sup>5</sup> Tager, M., and Hales, H. B., *J. Immunol.*, 1948, **60**, 1.

<sup>6</sup> Kaplan, M. H., and Spink, W. W., *Blood*, 1948, **3**, 573.

pseudoglobulin and albumin are devoid of inhibitor, as are the pseudoglobulin of chicken and albumin of horse plasma. Confirming our earlier work,<sup>3</sup> the albumins are found to contain most of the cofactor. Positive results are obtained, however, with euglobulin of horse and pseudoglobulin of horse and chicken (weak). Negative results are found only with guinea-pig pseudoglobulin and chicken euglobulin. Guinea-pig euglobulin could not be tested with certainty owing to fibrinogenolysis. Thrombin interferes with tests on euglobulin in similar experiments with human and some other species of plasma.

**Summary.** Marked species variations in the staphylocoagulation phenomenon are encountered and can be explained in terms of several factors. Comparing with rabbit plasma as an empirical standard (shortest staphylocoagulation times encountered), the ability to shorten staphylocoagulation time by addition of cofactor (human serum albumin) is evidence for lesser amounts of cofactor in such plasmas as horse and bovine. Cofactor cannot be recovered completely or uniformly in plasma fractions obtained with the classical  $(\text{NH}_4)_2\text{SO}_4$  salting procedures. Nevertheless, the "albumin" is consistently rich in cofactor (for activation of prostaphylocoagulase) and de-

void of thrombin (which interferes in tests on some euglobulins).

Excess of inhibitor(s), in part at least directed against active staphylocoagulase as shown by pre-incubation of prostaphylocoagulase with cofactor before adding to plasma (or plasma dilution + fibrinogen) is a feature of some plasmas, notably rat and chicken. Owing to this, the demonstration of cofactor requires plasma fractionation, in these instances.

Fibrinogenolytic and possibly other proteolytic effects may interfere with tests, especially at higher ( $37^\circ\text{C}$ ) temperatures, and are exemplified in guinea-pig and dog plasmas.

The quantitative evaluation of staphylocoagulation times must, therefore, take cognizance of variability not only in the two bacterial factors but, even more significantly, in the various plasma factors on which they operate in the systems devised for testing the staphylocoagulation and proteolytic<sup>9</sup> phenomena. There are marked species differences in these plasma factors.

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<sup>9</sup> Gerheim, E. B., and Ferguson, J. H., *Proc. Soc. Exp. Biol. and Med.*, following paper.

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### 17156. Species Reactivity to Staphylokinase.\*

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The mechanism of staphylococcal fibrinolysis has been shown recently<sup>1,2</sup> to be anal-

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<sup>1</sup> Lack, C. H., *Nature*, 1948, 161, 559.

<sup>2</sup> Gerheim, E. B., Ferguson, J. H., Travis, B. L., Johnston, C. L., and Boyles, P. W., *Proc. Soc. Exp. Biol. and Med.*, 1948, 68, 246.

ogous to the mechanism of streptococcal fibrinolysis,<sup>3</sup> namely, a kinase type of activation by the bacterial product, of plasma or serum proenzyme (protease precursor) to yield the active lytic agent. In an earlier study,<sup>2</sup> of which the present data are an extension, the distinction between the bacterial factor taking part in proteolysis (*staphylokinase*) and that involved in coagulation (*prostaphylocoagulase*)<sup>4</sup> was made by means of

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<sup>3</sup> Milstone, H., *J. Immunol.*, 1941, 42, 109.

<sup>4</sup> Gerheim, E. B., and Ferguson, J. H., *Proc. Soc. Exp. Biol. and Med.*, previous paper.

TABLE III.  
Tests for Inhibitor and Cofactor.

Species plasma	Inhibitor			Cofactor		
	Euglob.	Pseudoglob.	Alb.	Euglob.	Pseudoglob.	Alb.
Chicken	+	0	+	0	±	+
Horse	±	+	0	+	+	+
Guinea pig	?	0	0	?	0	+
	(lysis)			(lysis)		

tion of thrombin after 24 hours, fibrinogenolysis is not significant in these cases: (E) With guinea-pig plasma, our findings are staphylocoagulation at 20°C in a time which is the same whether or not cofactor is added. The last is not a variable, therefore. Nevertheless, at 37°C, clots obtained in the presence of cofactor are not quite solid and undergo fibrinolysis subsequently. Without cofactor, no clot is obtained at 37°C and the 24 hr. test with thrombin is negative, showing that fibrinogenolysis has occurred. Our experiments are a partial confirmation of those of Smith and Hale<sup>1</sup> but complete the explanation in terms of interfering effects of proteolysis induced by the accompanying staphylokinase<sup>7,8</sup> factor in the staphylococcal product, with no need to postulate any instability of the staphylocoagulase. The fact that in guinea-pig plasma staphylocoagulation is faster at 37° than at 20° confirms Kaplan and Spink,<sup>6</sup> but these workers' data are probably more significant as to temperature coefficient since their staphylococcal product would appear to have been deficient in staphylokinase: (F) in dog plasma, proteolysis, at 37°, is also encountered but the staphylokinase interference is less, so that some staphylocoagulation still occurs. That the clotting is slower at the higher temperature, in this case, does indicate greater interference by the proteolysis as the temperature is raised from 20° to 37°C. Moreover, it is slower, at 37°C, when cofactor is not added. Owing to interference by fibrinogenolysis it can be suggested only tentatively that dog plasma has more cofactor than guinea-pig's and less than rabbit's.

*Inhibitory effects of various plasmas on staphylocoagulation.* In order to investigate further the inhibitory phenomena, the special tests summarized in Table II were performed on plasmas selected from chicken, horse, guinea-pig, *i.e.* representative, respectively, of 1. antistaphylocoagulase, 2. inhibitor accompanying lesser amount of cofactor, 3. lesser cofactor with accompanying proteolysis. Tests were performed at 37° C on mixtures of fibrinogen (bovine, 1 ml), cofactor (human albumin, 0.5 ml), and prostaphylocoagulase (0.25 ml). To this 0.25 ml of various plasma dilutions was added. As compared with a 40 minutes staphylocoagulation time in the control (buffer instead of plasma), definite inhibitory effects were apparent in all cases and lessened on dilution. The inhibitor is highest in undiluted chicken plasma. Proteolytic effects did not significantly interfere when guinea-pig plasma was tested under these conditions.

*Cofactor and inhibitor of staphylocoagulation in various plasma fractions:* The euglobulin, pseudoglobulin and albumin fractions of the plasmas of the above three species were tested: 1. in a repetition of the foregoing demonstration of inhibitor, using only the full strength plasma fraction, however; 2. in a similar test system but omitting the human albumin (cofactor) in order to detect the presence of cofactor in the various plasma fractions. The data are given, qualitatively, in Table III.

We have noted that no fraction yields an inhibitor concentration comparable to that of the original plasma and, in fact, the total inhibition by all fractions does not equal that of the whole original plasma. Owing to proteolysis, it is not possible to be certain of the inhibitory effect of guinea-pig euglobulin. Its

<sup>7</sup> Gerheim, E. B., Ferguson, J. H., Travis, B. L., Johnston, C. L., and Boyles, P. W., *Proc. Soc. Exp. Biol. and Med.*, 1948, **68**, 246.

<sup>8</sup> Laek, C. H., *Nature*, 1948, **61**, 559.

vate rabbit plasma with his staphylokinase preparations and hence suggested possible plasma inhibitors.

The working out of quantitative methods to estimate and distinguish between plasma differences as to enzyme precursor, on the one hand, and the possible inhibitors, on the other, is beyond the scope of the present communication.

It is, nevertheless, possible to conclude that, in addition to individual plasma variations, there are wider and more significant species variations, clearly illustrated by the data of Table I, which, whatever the ultimate cause, do represent definite species differences in the plasma reactivity of staphylokinase.

Staphylokinase fibrinolysis in human plasma Fraction-I was noted in a previous paper.<sup>2</sup> Clearly, staphylokinase and streptokinase can only be compared on human plasmas or products containing the species reactive proenzyme and equivalent in terms of such still ill-defined factors as the possible antikinase and antiprotease inhibitors.

*Rates of activation of proenzyme by staphylokinase and streptokinase.* Numerous experiments in our laboratory confirm the conclusion of previous workers<sup>11,12</sup> that streptokinase activates human plasma proenzyme "almost instantaneously" (Kaplan, 1945). In such case, attention is directed away from the fact that the events following the addition of bacterial kinase to plasma clot systems occur in two phases, namely, *first* the activation of proenzyme to lytic agent, and only *secondly* lysis of the substrate (fibrin). Early in our studies of staphylokinase the question was raised whether this activator might not differ from streptokinase in the rapidity of the first phase reaction. The following test system was, therefore, devised to permit independent following of the progress of the activation of proenzyme. Serum (4 ml) was used as a fibrinogen-free source of proenzyme and incu-

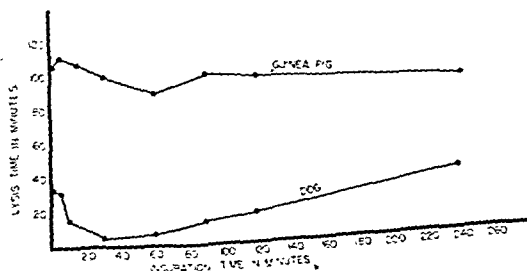


FIG. 1.

Activation of serum proenzyme by staphylokinase.

bated at 37°C with the staphylococcal product (1 ml). After suitable incubation periods, 0.5 ml of the mixture was added to 1 ml proenzyme-free bovine fibrinogen and immediately clotted with 0.5 ml thrombin. Such experiments were performed on human, rabbit, guinea-pig, and dog sera. Typical results from sera of the last two species are recorded in Fig. 1. It may be seen that the shortest lysis times (optimal protease activity) required an incubation averaging 30-60 minutes. In the experiment with dog serum, the lysis test after 15 seconds incubation was 33 minutes, but reached an optimal of 4 minutes lysis time after incubation for half an hour. Thereafter, the lysis times gradually lengthened, reaching 36 minutes after 4 hours of incubation, obviously denoting instability or inactivation of the enzyme at the 37° temperature. The guinea-pig data are less striking but qualitatively similar.

*Summary.* Fibrinolytic tests are described which clearly demonstrate differences in the species reactivity of plasmas to the staphylokinase and streptokinase activation of the protease system. Whereas streptokinase acts only on human materials, staphylokinase works on plasma (or serum) of dog, guinea-pig, and rabbit also, but gives negative results with bovine and other species tested. Staphylokinase differs from streptokinase also in requiring an incubation period of many minutes before reaching optimal activation of the serum proenzyme.

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<sup>11</sup> Kaplan, M. H., *Proc. Soc. Exp. Biol. and Med.*, 1944, 57, 40.

<sup>12</sup> Christensen, L. T., and MacLeod, C. M., *J. Gen. Physiol.*, 1945, 23, 559.

TABLE I.  
Staphylococcal Fibrinolysis and Inhibition by Crystalline Soybean Trypsin-inhibitor, in Plasma Clots of Various Species. 37°C; pH = 7.7 (borate buffer).

Species	I. Staphylokinase	II. Staph. + inhibitor
Dog	25 min.	60 min.
Human	50 min.	165 min.
Guinea pig	60 min.	225 min.
Rabbit	270 min.	No lysis (48 hr)
Ox	No lysis (72 hr)	No lysis (72 hr)
Horse	"	"
Sheep	"	"
Rat	"	"
Chicken	"	"

heat lability experiments. The current study concerns (1) species differences in plasma factors which react with staphylokinase, (2) differences between staphylokinase and streptokinase (a) in these species plasma reactivities and (b) in the rates with which they induce their respective activation of protease precursor.

**Reagents.** 1. Plasmas were collected from the various animal species by centrifugation of blood received into one-tenth volume of 4% sodium citrate (hydrated); 2. Thrombin was the commercial (Parke, Davis) "Thrombin Topical" diluted to 10-15 units/ml; 3. Fibrinogen was Armour's bovine plasma Fraction-I, which was proenzyme-free<sup>5</sup> inasmuch as no active protease could be detected after treatment with (1) chloroform, (2) bacterial kinases, or (3) "fibrinokinase" of Astrup and Permin;<sup>6</sup> 4. The staphylococcal product, containing both *staphylokinase* and *prostaphylocoagulase* (of no consequence in the presence of added thrombin) was prepared in 1% solution as previously described;<sup>7</sup> 5. Crystalline soybean antiprotease (trypsin-inhibitor), courtesy of Dr. M. Kunitz,<sup>8</sup> was used in 1:10,000 solution; 6. Borate buffer,<sup>9</sup> pH = 7.7, was used as solvent and diluent throughout.

*Staphylokinase- and streptokinase-induced fibrinolysis in plasma clots of various species.*

<sup>5</sup> Ferguson, J. H., Travis, B. L., and Gerheim, E. B., *Proc. Soc. Exp. Biol. and Med.*, 1947, **64**, 285.

<sup>6</sup> Astrup, T., and Permin, P. M., *Nature*, 1947, **159**, 681.

<sup>7</sup> Gerheim, E. B., Ferguson, J. H., and Travis, B. L., *Proc. Soc. Exp. Biol. and Med.*, 1947, **66**, 525.

<sup>8</sup> Kunitz, M., *J. Gen. Physiol.*, 1946, **29**, 149.

Confirming earlier workers,<sup>9</sup> we find *streptokinase* to lyse human plasma (fibrin) clots, but unable to induce fibrinolysis in plasma of other animal species.

*Staphylokinase* is tested in the following systems: I. 0.5 ml citrated plasma + 0.25 ml staphylokinase + 0.25 ml buffer + 0.25 ml thrombin; II. similar mixtures but containing 0.25 ml 1:10,000 crystalline soybean antiprotease instead of buffer. The data given in Table I are representative of a number of experiments.

Staphylokinase does not show the strict species specificity of streptokinase, but does fail to lyse plasma clots (Table I) of ox, horse, sheep, rat, and chicken. Fibrinolysis is obtained, however, with dog, human, guinea-pig, rabbit. Moreover, the respective lysis times are usually in that order both in I. and in II. In the latter series, a weak protease inhibitor is chosen to bring out its relative inhibitory effects and these show a definite correspondence with the initial enzyme potency. Thus, in the case of the weak rabbit protease the inhibitor inhibits fibrinolysis for at least 48 hours, whereas its effect is relatively minor, i.e. 60 min. vs. 25 min., on the lysis time in dog plasma tests.

In repeated tests on a single species, e.g. rabbit plasma, considerable variations in lysis time are encountered, despite the use of the same staphylokinase preparation and carefully standardized test conditions. It may be recalled that Lack<sup>10</sup> consistently failed to acti-

<sup>9</sup> Tillett, W. S., and Garner, R. L., *J. Exp. Med.*, 1933, **58**, 485.

<sup>10</sup> Lack, C. H., *Brit. J. Exp. Pathol.*, 1948, **29**, 191.

It is apparent that the experimental data are not consistent, and do not permit general conclusions as to the significance of the spreading factor in the growth mechanism of malignant tumors. In the present work we have investigated the effect of various testis extracts on the growth of a transplanted mouse lymphosarcoma. We have also considered the relationship of the principle affecting the growth of tumors to the Duran-Reynals spreading factor, identified with the enzyme hyaluronidase.

*Material and methods.* The experimental work has been done with mice of the AKm stock. The tumor used was derived from the 9417 strain of lymphatic leukemia, described by Burchenal, Lester, Riley and Rhoads.<sup>12</sup> This leukemia is of spontaneous origin. According to Willis<sup>13</sup> we have used the term "lymphosarcoma" for the localized growth of the disease. The tumor was propagated by subcutaneous implants into the right side of the abdomen of the mice. A piece of tumor was minced with scissors in physiological saline solution, filtered through cotton, and the cell suspension diluted to contain 1 million cells in 0.1 ml.

In the experimental animals 0.1 ml (1 million cells) of the tumor suspension was mixed with 0.1 ml of testis extract and inoculated at once into the mice. The control animals were given 0.1 ml of the suspension of malignant cells mixed with 0.1 ml of physiological saline solution. In the control animals a tumor developed at the site of injection after 8-12 days. Successively it attained the size of 3-4 cm in diameter, infiltrated the abdominal musculature and peritoneum, became necrotic, hemorrhagic and finally ulcerated. The homolateral inguinal and axillary lymph nodes were always involved. The disease usually spread to the internal organs before the animals died on an average of 30 days following the inoculation.

We have used 3 preparations of testis extract from AKm mice designated "crude

extract", "granule fraction", and "supernate fraction". All of these represented a concentration of 50% in relation to the amount of testis tissue employed. The "crude extract" was prepared by grinding the testis tissue with equal amounts of distilled water and centrifuging once for 20 minutes at 600 x g. The resulting supernate constituted the "crude extract." The "granule fractions" were prepared by grinding testis tissue with equal amounts of either distilled water or 30% sucrose solution. The homogenates were centrifuged 3 times for 20 minutes at 600 x g and the sediments discarded. The supernates were then centrifuged 30 min. at 20,000 x g, and the sediments consisting of granules staining with Janus Green B, washed once or twice in either distilled water or 30% sucrose solution. After the final centrifugation at 20,000 x g, the sediments were taken up in distilled water, and the fractions designated "granule, water" and "granule, sucrose". Because of the observation of Hogeboom, Schneider and Pallade<sup>14</sup> that the large granules of the cytoplasm (mitochondria) were preserved best in 30% sucrose solution, we found it necessary to compare the activity of the "granules" prepared in distilled water with those prepared in sucrose solution. Since it was not considered desirable to use hypertonic sucrose solution for injection purposes, we used only distilled water for the final suspension. The "supernate fraction" represented the supernate from the first high speed centrifugation of a "granule fraction" prepared with distilled water. This supernate was purified by one or two more centrifugations at high speed.

In preliminary experiments we have investigated the content in the testis extracts of the factor, supposed to be hyaluronidase,

TABLE I.  
Showing the Effects of Various Testis Extracts on the Intradermal Spread of India Ink.

Extracts	Spreading coefficient
Crude extract	21
Supernate fraction	21
Granule, water	3
Granule, sucrose	3
Control	1

<sup>12</sup> Burchenal, J. H., Lester, R. A., Riley, J. B., and Rhoads, C. P., *Cancer*, 1948, 1, 399.

<sup>13</sup> Willis, R. A., *Pathology of Tumours*, Mosby, 1948.

<sup>14</sup> Hogeboom, G. H., Schneider, W. C., and Pallade, G. E., *J. Biol. Chem.*, 1948, 172, 619.



# 17157. Effect of Testis Extract on Growth of a Transplanted Lymphosarcoma in AKm Mice.\*

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(Introduced by C. P. Rhoads).

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Mammalian testis extract, as well as many bacteria, contains a factor which enhances the *in vivo* spread of dyes, infective organisms and toxins. This substance is accepted to be identical with the enzyme hyaluronidase.<sup>1</sup> Since the first description of the spreading factor by Duran-Reynals,<sup>2</sup> a number of workers have considered the possible relationship between this factor and the local and distant spread of malignant growths. Such investigations have sought to determine, 1) the actual content of spreading factor in tumors, as demonstrated by *in vivo* and *in vitro* methods, and 2) the behaviour of tumor transplants under the influence of the factor. Duran-Reynals and Stewart<sup>3</sup> demonstrated the spreading factor in some human carcinomas, but not in sarcomas. McCutcheon and Coman<sup>4</sup> also offered evidence of the spreading factor in human carcinomas. In a series of animal tumors Boyland and McClean<sup>5</sup> found a great variation in spreading activity, with the highest values in mouse and fowl sarcomas. The concentration of spreading factor in tumors never approached that of normal testis tissue. Hakanson and Glick<sup>6</sup> reported an ele-

vation in the anti-hyaluronidase titer in sera from patients with malignant tumors. This increase was more marked in cases of metastatic than of non-metastatic tumors. Coman, McCutcheon and Zeidman<sup>7</sup> gave several injections of hyaluronidase in or around transplanted mouse Sarcoma 241, and Shope Papilloma, but observed no effect on the growth of the tumors.

When the malignant transplants were combined with testis extract prior to inoculation, Duran-Reynals<sup>8</sup> found that the growth of the Brown-Pearce carcinoma was retarded or prevented. In his discussion he favors the explanation that this phenomenon is due to the increased cellular and tissue permeability brought about by the spreading factor. He states, however, that this unitarian interpretation of the effects of testis extract is not positively established. Using a similar technique, Prime and Haagensen<sup>9</sup> could not demonstrate any effect on a transplanted rat sarcoma, a rat carcinoma or a guinea pig sarcoma. Hoffman, Parker and Walker<sup>10</sup> were able to show that rabbit testis extract enhanced the spread of the Chicken Tumor I, while Tanzer<sup>11</sup> found that testis extract inhibited the growth of mouse Sarcoma 180, but was without effect on Sarcoma 37 and the Bashford Adenocarcinoma. Tanzer suggests that the testis extract may either injure the cells of the tumor, or increase the resistance of the inoculated animal, and that the Duran-Reynals spreading factor may or may not, be identical with the factor acting upon tumors.

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<sup>1</sup> Meyer, Karl, *Physiol. Rev.*, 1947, **27**, 335.

<sup>2</sup> Duran-Reynals, F., *J. Exp. Med.*, 1929, **50**, 327.

<sup>3</sup> Duran-Reynals, F., and Stewart, F. W., *Am. J. Cancer*, 1931, **15**, 2790.

<sup>4</sup> McCutcheon, M., and Coman, D. R., *Cancer Res.*, 1947, **7**, 379.

<sup>5</sup> Boyland, E., and McClean, D., *J. Path. and Bact.*, 1935, **41**, 553.

<sup>6</sup> Hakanson, E. Y., and Glick, D., *J. Nat. Cancer Inst.*, 1948, **9**, 129.

<sup>7</sup> Coman, D. R., McCutcheon, M., and Zeidman, I., *Cancer Res.*, 1947, **7**, 383.

<sup>8</sup> Duran-Reynals, F., *J. Exp. Med.*, 1931, **51**, 493.

<sup>9</sup> Prime, F., and Haagensen, C. D., *Am. J. Cancer*, 1934, **20**, 630.

<sup>10</sup> Hoffman, D. C., Parker, F., and Walker, T., *Am. J. Path.*, 1931, **7**, 523.

<sup>11</sup> Tanzer, R. C., *J. Exp. Med.*, 1932, **55**, 455.

of the "supernate" is 7 times that of the "granule fraction". In contrast the tumor influencing activity in the "granule fraction" is slightly higher than that of the "supernate fraction". (Table III). These findings indicate that the "lymphosarcoma factor" is not identical with the spreading factor (hyaluronidase).

*Summary.* A factor has been demonstrated in testis extract which inhibits the growth of a transplanted lymphosarcoma in AKm mice.

This factor is of unknown nature, but appears to be different from the Duran-Reynals spreading factor.

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### 17158. Effects of Urethane on Living Tissue.\*

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Ethyl Carbamate (urethane) has been shown to be a useful agent in the treatment of wound infections.<sup>1,2</sup> When extensive wounds or large areas of intact skin are exposed to the prolonged application of 10% urethane solution<sup>2</sup> about 20 to 30% of the patients treated develop nausea and vomiting. This suggests that the drug is absorbed through open wounds and normal skin surfaces. The nausea and vomiting reported by Howe and Weinstein,<sup>2</sup> and Howe<sup>1</sup> in urethane treated wound infections and by Paterson *et al.* in leukemia patients receiving the drug by mouth<sup>3</sup> appeared to be benign in nature. However, because the treatment of human wound infections requires prolonged exposure to large amounts of this drug, experiments were conducted to investigate its effect on the organs of animals treated with similar doses.

A group of 10 guinea pigs was given lethal amounts of urethane administered intraperitoneally in rapidly ascending doses over a short period of time (Table I, Pigs 1-10). The gross and histological tissue changes were compared with those of a second group of 10

animals receiving daily subcutaneous injections of urethane in dosages simulating those used in the topical treatment of infected wounds in man (Table II, Pigs 14-23). Hypnotic effects ranging from drowsiness and ataxia to terminal anesthesia were noted. Some animals recovered and ate heartily after 8 hours of complete anesthesia. In the treatment of a large infected wound a person is exposed to a maximum of 60 g of urethane per day, although only a fraction of this is absorbed due to loss in dressings. The animals in Group II received 0.1 g per 100 grams of body weight per day for 28 consecutive days which is about twice as long as the average duration of therapy in 39 urethane treated patients.<sup>2</sup> This is comparable to the maximal dosage used in man, and it was given subcutaneously to insure complete absorption. Dosages were corrected weekly for fluctuations in weight. Control animals (Table I, Pigs 11-13; Table II, Pigs 24-26) received corresponding amounts by volume of normal saline. Diet consisted of Purina chow, lettuce and cabbage. All animals in Group II gained weight and at the end of the experiment were healthy and had lustrous silky coats. They were killed on the 29th day.

Examination of the organs was made immediately after death. Sections were fixed in 5% formaldehyde solution, imbedded in paraffin and stained with hematoxylin eosin. Brain, striated muscle, myocardium, lungs,

\* This study was aided by the President's Fellowship of Brown University.

<sup>1</sup> Howe, C. W., *Surg., Gynec. and Obst.*, 1948, **87**, 425.

<sup>2</sup> Howe, C. W., and Weinstein, L., *Surg., Gynec. and Obst.*, 1947, **84**, 913.

<sup>3</sup> Paterson, E., Thomas, I. A., Haddow, A., and Watkinson, J. M., *Lancet*, 1946, **1**, 677.

TABLE II.  
Showing the Growth of the Lymphosarcoma as Influenced by Testis Extract.

Inoculum	Total No.	No. with tumor	No. without tumor
Lymphosarcoma + testis extr.	99	55 (55.5%)	44 (44.5%)
Lymphosarcoma + physiol. saline	43	39 (91%)	4 (9%)

TABLE III.  
Showing the Relative Effect of Different Testis Fractions on Growth of the Lymphosarcoma.

Inoculated with lymphosarcoma +	Total No.	No. with tumor	No. without tumor
Crude extr.	39	18 (46%)	21 (54%)
Granule fraction	32	19 (59%)	13 (41%)
Supernate "	28	18 (64%)	10 (36%)
Physiological saline	43	39 (91%)	4 (9%)

which affects the spread of India Ink injected intradermally. Testis extracts were combined with equal amounts of India Ink, diluted 1:5 with distilled water, and 0.4 ml of the mixtures injected intradermally on the shaved back of albino rabbits. India ink plus distilled water served as control. After 24 hours the longitudinal (D) and latitudinal (d) extent of the spread were measured and the area of spreading estimated according to the formula  $D \times d$

$\pi \frac{D \times d}{4}$ .<sup>15</sup> The figure representing the spread of the extract, divided by that of the control, was designated the spreading coefficient.

**Results.** Table I shows the spread of the India Ink as influenced by the various fractions of testis extract.

As shown in Table I both the "crude extract" and the "supernate fraction" gave very marked spreading effects. On the other hand the "granule fraction" produced a spread of India Ink which was small and hardly significant. It is emphasized that the "granules" gave the same spread, whether they were prepared with distilled water or with 30% sucrose solution.

Tables II and III present the effect of testis extract on the transplanted lymphosarcoma in 99 mice. There were 43 controls. The whole group of animals given testis extract is presented without regard to the particular fractions of the extract in Table II, while

Table III relates the effects of the individual fractions.

In the control group 9% of the animals do not develop tumors, whereas, in the group treated with testis extract, 44.5% fail to take the transplant. This difference is statistically significant. Some of the test animals were killed when the controls died. On autopsy slightly enlarged subcutaneous lymph nodes were found on the same side as the injections. The spleens were small, and there were no macroscopical signs of metastasis to other inner organs. Most of the animals, however, were allowed to live 2 months or more after the inoculation, and appeared to remain in good health.

Table III indicates the relative effect of the "granule" and "supernate" fractions of testis extract on the growth of the lymphosarcoma.

The "crude extract" showed slightly greater activity than the more purified fractions. Interestingly the "granule" and "supernate" fractions seemed to have practically the same activity, with a slight difference in favor of the "granule fraction". The "granules" showed the same effect whether they were prepared with 30% sucrose or distilled water.

If the principle affecting the growth of this lymphosarcoma was identical with the spreading factor (hyaluronidase), we would expect to demonstrate a significant difference between the activity of the "granule" and "supernate" fractions, a difference comparable to that demonstrated in the spreading coefficients (Table I). As shown there the spreading coefficient

<sup>15</sup> Madinaveitia, J., *Biochem. J.*, 1938, **32**, 1806.

The tissues at the site of injection revealed blood pigment and recently extravasated blood cells in the subcutaneous tissues. Independently from these hemorrhagic changes, a mesenchymal reaction consisting of histiocytes and lymphocytoid cells was found in a number of guinea pigs.

Microscopic sections of the lungs in two control animals showed engorgement of blood vessels, mural edema, minimal endothelial damage and red cell extravasations. In the same two animals there were dilated hepatic sinusoids with red cell extravasations and increased cellularity in the pulp substance of the spleen.

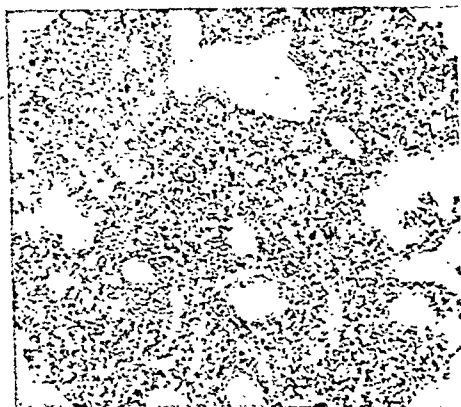


Fig. 1.

Diffuse septal mesenchymal cell infiltration in lung of a guinea pig;  $\times 25$ .



Fig. 2.

Pulmonary artery and vein displaying perivascular edema and mesenchymal reaction:  $\times 125$ .

**Discussion.** The changes in Group I animals which died from lethal doses of urethane were not significantly greater than those in Group II which were killed in apparent health, to explain the death of the former on a morphologic basis. It is our opinion that death occurred through disturbance of the respiratory mechanism as a result of the central hypnotic action of the drug and that the tissue changes in Group II were compatible with healthy life.

Vascular dilation and engorgement in the lungs, liver and spleen at times accompanied by mural changes were consistently seen. The frequent finding of areas of perivascular edema or hemorrhage without evidence of damage to vessel walls might indicate a reversibility of these mural changes. Vascular injury comparable to the mildest forms seen in the urethane treated animals were also observed in two of the saline treated control animals. Although urethane induced renal damage has been reported in specific strains of mice,<sup>4,5</sup> the glomerular apparatus in our guinea pigs remained essentially intact.

The hepatic changes compare with those previously reported by Doljanski and Rosin<sup>6</sup> in a study of urethane treated rats. The cellular damage consisted mainly of cytoplasmic vacuolization, a condition met with under a variety of pathological states. This condition has been produced by perfusion with 0.6 NaCl Solution<sup>7</sup> and Ringer's Solution<sup>8</sup> and vacuolization has been noted as early as 10 minutes after perfusion started. Raum<sup>7</sup> considered it a reversible phenomenon.

A striking reaction of cellular mesenchyma was consistently found in both groups of animals, most apparent in the lungs and spleen. A similar proliferation of mesenchy-

<sup>4</sup> Dunn, T. B., and Larsen, C. D., *Fed. Proc.*, Part II, 1946, 5, 220.

<sup>5</sup> Kirschbaum, A., and Bell, E. T., *Proc. Soc. Exp. Biol. and Med.*, 1947, 64, 71.

<sup>6</sup> Doljanski, L., and Rosin, A., *Am. J. Path.*, 1944, 20, 945.

<sup>7</sup> Raum, J., *Arch. f. Exp. Path. u. Pharmacol.*, 1902, 29, 353.

<sup>8</sup> von Skromlik, E., and Hunnerman, T., *Z. f. d. ges. exp. Med.*, 1920, 11, 349.

TABLE I.  
10% Urethane Intraperitoneally in Lethal Doses.

Pig No.	Wt in g	Days treated	No. inj.	Total dose, g	Fate
1	248	9	5	0.99	Died
2	250	13	6	1.32	"
3	316	13	6	1.47	"
4	267	9	5	1.02	Killed
5	269	6	4	0.83	"
6	842	3	3	7.57	Died
7	821	3	3	7.81	"
8	906	3	3	8.19	"
9	757	3	3	6.80	"
10	842	3	3	7.58	"
11*	757	3	3	0.58†	Killed
12*	250	10	6	0.11†	"
13*	270	9	5	0.09†	"

\* Saline treated control.

† Grams of NaCl.

TABLE II.  
10% Urethane Subcutaneously.

Pig No.	Initial wt, g	Total No. daily inj.	Total dose, g	Final wt, g
14	442.0	28	13.81	581.6
15	353.2	20*	8.19	468.4
16	396.7	20*	8.46	393.4
17	344.5	28	10.47	432.1
18	412.9	28	13.23	528.3
19	399.0	28	12.44	492.4
20	355.5	28	10.96	412.2
21	391.2	28	12.19	503.7
22	441.5	28	13.82	556.6
23	274.1	28	9.67	440.5
24†	434.4	28	1.18‡	563.7
25†	392.4	28	1.10‡	558.1
26†	390.4	28	1.18‡	514.1

\* Injections omitted after 20th day due to development of pneumonia. Animal recovered.

† Saline treated control.

‡ Grams of NaCl.

liver, spleen, pancreas, kidneys, adrenals and gonads were examined.

**Findings.** Only the significant changes are described. In the group of animals submitted to intraperitoneal injections of lethal doses of urethane (Group II) there was congestion in the lungs, liver and spleen and occasionally in the kidneys. Vascular changes characterized by endothelial swelling and desquamation, mural edema and extravasation of plasma and formed blood elements into the surrounding tissues were occasionally present. Cytoplasmic vacuolization was observed in the hepatic cells and glandular epithelium of the renal tubules. A striking hyperplasia of mesenchymal cells was noted in the lungs and spleen and to a lesser degree in the myocardium and liver. Frequent hepatic cell changes consist-

ing of an increase in fat droplets, cytoplasmic swelling and rarefaction were present. The central lobular cells in 3 animals<sup>3,8,10</sup> displayed loss of cellular outlines. The kidneys revealed no glomerular damage. There were hemorrhages in the cortex and medulla in animals 2 and 9.

In the animals subjected to sublethal injections of urethane over long periods of time (Group II), the striking feature was a mesenchymal cell reaction in the lungs and spleen. Cytoplasmic vacuolization of the hepatic cells was frequently seen. Vascular engorgement in the lungs, liver and spleen often accompanied by mural edema and by escape of plasma and formed blood elements were occasionally encountered.

The kidneys showed no significant findings.

TABLE I.

Antagonistic Action of 2-substituted-1,3-propanediols to the Lethal Effects of Metrazol 100 mg per kg. Metrazol and one of the other drugs were given simultaneously by intraperitoneal injection to white mice.

$\text{CH}_2\text{OH} - \begin{array}{c} \diagup \text{R}_1 \\ \text{C} - \text{OH} \\ \diagdown \text{R}_2 \end{array} - \text{CH}_2\text{OH}$			
No.	R <sub>1</sub>	R <sub>2</sub>	Mean protective dose in mg per kg
1	methyl	methyl	>250
2	ethyl	ethyl	40
3	n-propyl	n-propyl	88
4	n-butyl	n-butyl	>250
5	methyl	ethyl	135
6	"	n-propyl	70
7	"	iso-propyl	88
8	"	n-butyl	210
9	ethyl	iso-propyl	77
10	"	ethoxy	>250
11	phenyl	H	>250
12	"	ethyl	120
13	phenoxy	"	>250
14	phenobarbital		39
15	myanesin		80

DEP resembled myanesin in causing transient paralysis of skeletal muscles. The paralyzing activity of DEP was, however, of a lower order than that of myanesin, the doses causing loss of the righting reflex in 50% of mice after intraperitoneal administration being 390 mg per kg and 180 mg per kg, respectively. In small doses not causing paralysis DEP somewhat increased the spontaneous activity of the animals whereas myanesin had a sedative effect. The acute toxicity of DEP was very low. The mean lethal dose in mice on intraperitoneal administration was 1400 mg per kg. Doses as high as 30 mg per kg given intravenously to anesthetized cats did not affect blood pressure and respiration. Similar doses did not alter normal electroencephalographic patterns in cats and did not influence spinal reflexes. On a weight for weight basis DEP was as active as phenobarbital in preventing the occurrence of convulsions and deaths from lethal doses of metrazol. It was more effective than myanesin, phenobarbital or other drugs in antagonizing the lethal and convulsant effects of strychnine.

DEP was more effective against convulsions

induced by chemical agents than against those induced by electric current. It abolished the tonic extensor component of supramaximal electroshock seizures in mice and rabbits but was in this respect about 6 times weaker than phenobarbital. The duration of the effect was also much shorter with DEP than with phenobarbital.

Myanesin is known to abolish abnormal electroencephalographic patterns in cases of true petit mal.<sup>3</sup> Because of the qualitatively similar anticonvulsant effect of DEP and myanesin, the stronger anticonvulsant action of DEP may be of value in the treatment of petit mal and of other disorders in which myanesin has been tried.

*Summary.* 2,2-Diethyl-1,3-propanediol and certain other substituted 1,3-propanediols possess qualitatively similar pharmacological properties as myanesin. These compounds differ from myanesin in having a weaker paralyzing action and a more powerful anticonvulsant action.

<sup>3</sup> Gammon, G. D., and Churchill, J. A., *Am. J. Med. Sci.*, 1949, **217**, 143.

mal cells is mentioned by Nettleship *et al.*<sup>9</sup> in urethane treated mice. Paterson *et al.*<sup>3</sup> reported fibrosis in the bone marrow, liver and spleen of urethane treated leukemia patients and the authors have noted excess fibroplasia in excised sections of urethane treated wounds.

It is conceivable that the induction of a degree of vascular dilation and excess fibroblastic proliferation would be beneficial in the treatment of healing wounds.

Although the evidence is insufficient to exclude a direct action of urethane on liver cells, it was productive of comparatively slight hepatic and renal damage even when given in

lethal doses. These changes are apparently causally dependent upon vascular injury which is reversible.

**Conclusions.** (1) Doses of urethane simulating those used in the topical treatment of infected wounds in man are not productive of toxic parenchymal changes in the organs of guinea pigs subjected to prolonged daily subcutaneous injections. The animals remained healthy and gained weight.

(2) A marked mesenchymal cell reaction was consistently seen in the lungs and to a lesser extent in the liver and myocardium of urethane treated animals.

<sup>9</sup> Nettleship, A., Henshaw, P. S., and Meyer, H. L., *J. Nat. Cancer Inst.*, 1943, **4**, 309.

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## 17159. Anticonvulsant Action of 2-Substituted-1,3-Propanediols.

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3-(*o*-Toloxyl)-1,2-propanediol, called myanesin, possesses two important pharmacological properties: it causes transient paralysis of skeletal muscles and has an anticonvulsant action.<sup>1</sup> The pharmacological examination of numerous related ethers of glycerol has shown that there was no simple relationship between their paralyzing and anticonvulsant activities. The observation that nuclear substitution of 3-phenoxy-1,2-propanediol markedly affected paralyzing activity but did not have any influence on anticonvulsant action<sup>2</sup> suggested the examination of the anticonvulsant properties of 2-substituted-1,3-propanediols.<sup>1</sup>

The anticonvulsant action of the compounds was determined in male, white mice, weighing

18 to 22 g. Metrazol 100 mg per kg, which caused convulsions and deaths in 99% of the animals, was injected intraperitoneally together with graded doses of the compound under test. Ten mice were used at each dose level. The animals were observed for 90 minutes following the injection and the incidence of convulsions and deaths noted. The relative efficacy of the substances was evaluated by finding graphically the dose protecting one half of the animals from convulsions and deaths. This mean protective dose of 13 experimental compounds and of phenobarbital and myanesin is given in Table I. Several of the investigated substances had anticonvulsant properties of a similar order as myanesin (No. 3, 6, 7, 9). Compounds containing butyl radicals (No. 4 and 8) were inactive. When a phenyl or phenoxy group was a substituent, activity was decreased or lost (No. 11, 12, 13). 2,2-Diethyl-1,3-propanediol, called DEP, possessed outstanding anticonvulsant properties. A short description of its pharmacological properties appears to be of interest.

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<sup>1</sup> Berger, F. M., and Bradley, W., *Brit. J. Pharmacol.*, 1946, **1**, 265.

<sup>2</sup> Berger, F. M., *J. Pharm. and Exp. Therap.*, 1948, **93**, 470.

† I am obliged to Mr. W. A. Lott of the Squibb Institute for Medical Research for supplying me with some of these compounds.

TABLE I.  
Serial Passages of Lansing Strain Virus in Hamsters.

Passage No.	No. of animals inoculated		No. of animals paralyzed		Incubation period in days	
	MDH	TF	MDH	TF	MDH	TF
1	4	4	2	4	3, 4	2, 3, 3, 5
2	5	4	2	3	3, 11	3, 3, 18
3	5	4	1	2	3,	4, 4
4	5	4	4	1	4, 4, 8, 9	4
5	4	4	1	2	8	5, 16
6	5	4	0	1	—	6
					Avg 5.7	Avg 5.1

TABLE II.  
Comparative Titrations.

Virus	Titrated in	LD <sub>50</sub> (in %)
MDH-hamster passage virus	MDH-hamsters	>10.0
	Cotton rats	5.0
	Mice	1.67
TF-hamster passage virus	TF-hamsters	.6
	Cotton rats	.018
	Mice	.054
Mouse-passage virus	MDH-hamsters	5.0
	TF-hamsters	.015
	Cotton rats	.004-.0008
	Mice	.0016-.0008
Cotton rat passage virus	MDH-hamsters	>5.0
	TF-hamsters	.09
	Cotton rats	.029-.009
	Mice	.028-.0015

animal, or of hamster passage virus, resulted in lower titers than when mice or cotton rats were used, and the lowest titers were obtained when hamster passage virus was titrated in hamsters.

*Attempts to increase the infectivity of the Lansing strain for the golden hamster.* Attempts were made to enhance the infectivity of the Lansing strain virus for TF-hamsters by using as diluent saline buffered at a low pH (technique of Hammon and Izumi<sup>5</sup>). In one experiment (with cotton rat passage virus) the result was better at pH 4, while in 2 other experiments, for which mouse-passage virus was used, better results were obtained at pH 7.2.

In 2 other experiments with TF-hamsters the technic described by Milzer and Byrd<sup>6</sup>

(autolyzed normal mouse brain tissue as adjuvant) was used. No enhancement of the infectivity was observed.

*Immune response.* It seemed interesting to investigate the immune response of the golden hamster to the Lansing strain virus. For this purpose, hamsters were immunized by intraperitoneal inoculations of .3 ml of a 10% suspension of live virus. MDH-hamsters were immunized with the mouse passage virus, while mouse passage, cotton rat passage and hamster passage virus were used for the immunization of 3 separate lots of TF-hamsters. The vaccinations were done twice weekly with a total of 7 vaccinations. In each lot, one group of animals was bled 2 weeks after the first vaccination, another group after 3 weeks, and still another group after 4 weeks (6 to 10 days after the last vaccination). The sera of each group were pooled and are referred to as 1st, 2nd and 3rd bleeding, respectively. The antibody content was determined by neutralization tests in mice.

TF-hamsters vaccinated with TF-hamster passage virus showed the weakest immune response with the maximum titer of 1:27. The titers of the sera of TF-hamsters vaccinated with mouse passage virus were 1:20 in the first bleeding, 1:47 in the second and 1:45 in the third bleeding. The vaccination of the MDH-hamsters with mouse passage virus gave a similar titer in the first bleeding (1:24) and a higher titer in the second bleeding (better than 1:96). There was a drop of the titer in the third bleeding.

The best results were obtained in TF-hamsters vaccinated with cotton rat passage virus. The titer was 1:17 in the first, better than 1:73 in the second, and better than 1:126 in the third bleeding.

<sup>5</sup> Hammon, W. McD., and Izumi, E. M., *Proc. Soc. Exp. Biol. and Med.*, 1941, 48, 579.

<sup>6</sup> Milzer, A., and Byrd, C. L., *Science*, 1947, 105, 70.



# 17160. The Golden Hamster (*Cricetus auratus*) as an Experimental Animal for Poliomyelitis Research.\*

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Successful passage of the Lansing strain of poliomyelitis virus to the Syrian or golden hamster was reported by Armstrong and Packchianian,<sup>1</sup> Plotz, Reagan and Hamilton,<sup>2</sup> and Durand.<sup>3</sup> The purpose of this study was to investigate how the golden hamster (*Cricetus auratus*) compares to the white mouse and the cotton rat as a laboratory animal for experimental work with the Lansing strain virus.

**Material and methods.** *Virus.* Suspensions of spinal cords and medullae of infected animals were made up in buffered saline (pH 7.2-7.4). Hamster-passage, mouse-passage and cotton-rat-passage virus was used.

*Hamsters.* Two strains of golden hamsters were used, one bred at the Michigan Department of Health (designated here as MDH-hamsters), the other obtained from the Tumblebrook Farm, Brant Lake, N. Y. (referred to as TF-hamsters).

**Comparative titrations and neutralization tests.** Five 5-fold dilutions were used in the comparative titrations. The numbers of animals for each dilution were: 4 for cotton rats and hamsters, and 6 for mice. All the neutralization tests were carried out in mice using constant amounts of mouse-passage virus and decreasing amounts of serum. The animals were observed for a period of 30 days in all the experiments.

**Titers and inocula.** The LD<sub>50</sub> titers of the virus preparations were calculated by the method of Reed and Muench<sup>4</sup> and expressed

in terms of dilution of the original tissue material (in %).

**Experimental. Serial passages.** Serial passages were carried out in MDH-hamsters as well as in TF-hamsters. Mouse-passage virus was used for the initial passage, followed by hamster-to-hamster passages. All the passages were done by intracerebral inoculation of 10% suspensions of cords and medullae.

As shown in Table I, the outcome of hamster-to-hamster passages did not show considerable differences between the 2 strains of hamsters, either in the percentage of takes or in the average incubation period. In the sixth passage in the MDH-hamsters none of the animals developed any symptoms. No attempt was made to continue the passages in TF-hamsters beyond the sixth passage in view of the low percentage of takes.

**Comparative virus titrations.** The outcome of the titrations of mouse passage, cotton-rat passage and hamster passage virus in mice, cotton rats and hamsters is summarized in Table II. The data presented show that the mouse passage and the cotton-rat passage virus gave extremely low LD<sub>50</sub> titers in MDH-hamsters (5% and more), while the same virus preparations gave high titers in cotton rats and mice (ranging from .028 to .0008% dilutions of virus). The MDH-hamster passage virus gave extremely low titers in MDH-hamsters (over 10%) as well as in cotton rats (5%) and mice (1.67%).

Considerably better results were obtained with TF-hamsters. Both mouse passage and cotton rat passage virus gave good titers in TF-hamsters (.015 and .09% respectively). The TF-hamster passage virus gave the lowest titer in TF-hamsters (.6%), but this titer was considerably higher than the corresponding titer obtained in MDH-hamsters (more than 10%). The general pattern was, however, the same with the 2 strains of hamsters: the use of the hamster as an experimental

\* The data presented in this paper were used by the senior author for a thesis in partial fulfillment of the requirements for the Master of Science degree at the Michigan State College.

<sup>1</sup> Armstrong, C., and Packchianian (unpublished data, see Vanderbilt Lectures, 1941).

<sup>2</sup> Plotz, H., Reagan, R., and Hamilton, H. L., *Proc. Soc. Exp. Biol. and Med.*, 1942, **51**, 124.

<sup>3</sup> Durand, P., *Compt. Rend. Soc. Biol.*, 1943, **139**, 716.

<sup>4</sup> Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27**, 493.

TABLE I.  
Serial Passages of Lansing Strain Virus in Hamsters.

Passage No.	No. of animals inoculated		No. of animals paralyzed		Incubation period in days	
	MDH	TF	MDH	TF	MDH	TF
1	4	4	2	4	3, 4	2, 3, 3, 5
2	5	4	2	3	3, 11	3, 3, 18
3	5	4	1	2	3,	4, 4
4	5	4	4	1	4, 4, 8, 9	4
5	4	4	1	2	8	5, 16
6	5	4	0	1	—	6
					Avg 5.7	Avg 5.1

TABLE II.  
Comparative Titrations.

Virus	Titred in	LD <sub>50</sub> (in %)
MDH-hamster passage virus	MDH-hamsters	>10.0
	Cotton rats	5.0
	Mice	1.67
TF-hamster passage virus	TF-hamsters	.6
	Cotton rats	.018
	Mice	.054
Mouse-passage virus	MDH-hamsters	5.0
	TF-hamsters	.015
	Cotton rats	.004-.0008
	Mice	.0016-.0008
Cotton rat passage virus	MDH-hamsters	>5.0
	TF-hamsters	.09
	Cotton rats	.029-.009
	Mice	.028-.0015

animal, or of hamster passage virus, resulted in lower titers than when mice or cotton rats were used, and the lowest titers were obtained when hamster passage virus was titrated in hamsters.

*Attempts to increase the infectivity of the Lansing strain for the golden hamster.* Attempts were made to enhance the infectivity of the Lansing strain virus for TF-hamsters by using as diluent saline buffered at a low pH (technique of Hammon and Izumi<sup>5</sup>). In one experiment (with cotton rat passage virus) the result was better at pH 4, while in 2 other experiments, for which mouse-passage virus was used, better results were obtained at pH 7.2.

In 2 other experiments with TF-hamsters the technic described by Milzer and Byrd<sup>6</sup>

(autolyzed normal mouse brain tissue as adjuvant) was used. No enhancement of the infectivity was observed.

*Immune response.* It seemed interesting to investigate the immune response of the golden hamster to the Lansing strain virus. For this purpose, hamsters were immunized by intraperitoneal inoculations of .3 ml of a 10% suspension of live virus. MDH-hamsters were immunized with the mouse passage virus, while mouse passage, cotton rat passage and hamster passage virus were used for the immunization of 3 separate lots of TF-hamsters. The vaccinations were done twice weekly with a total of 7 vaccinations. In each lot, one group of animals was bled 2 weeks after the first vaccination, another group after 3 weeks, and still another group after 4 weeks (6 to 10 days after the last vaccination). The sera of each group were pooled and are referred to as 1st, 2nd and 3rd bleeding, respectively. The antibody content was determined by neutralization tests in mice.

TF-hamsters vaccinated with TF-hamster passage virus showed the weakest immune response with the maximum titer of 1:27. The titers of the sera of TF-hamsters vaccinated with mouse passage virus were 1:20 in the first bleeding, 1:47 in the second and 1:45 in the third bleeding. The vaccination of the MDH-hamsters with mouse passage virus gave a similar titer in the first bleeding (1:24) and a higher titer in the second bleeding (better than 1:96). There was a drop of the titer in the third bleeding.

The best results were obtained in TF-hamsters vaccinated with cotton rat passage virus. The titer was 1:17 in the first, better than 1:73 in the second, and better than 1:126 in the third bleeding.

<sup>5</sup> Hammon, W. McD., and Izumi, E. M., *Proc. Soc. Exp. Biol. and Med.*, 1941, **48**, 579.

<sup>6</sup> Milzer, A., and Byrd, C. L., *Science*, 1947, **105**, 70.

**Discussion.** The two strains of golden hamsters used by the authors differ considerably in their susceptibility to the Lansing strain virus and in the titer of the hamster passage virus. One of the strains (MDH) is clearly not suitable for experimental research with the Lansing strain virus, and the other (TF) which gave considerably more favorable results, also is in every respect inferior to both the white mouse and the cotton rat.

The immunization of the hamster with active Lansing strain virus resulted in fairly good titers of neutralizing antibody, provided cotton-rat passage or mouse passage virus was used for immunization. In view of the small size of the hamster, use of this animal for serological work with the Lansing strain virus does not seem to present any particular advantage.

**Summary and conclusions.** Golden ham-

sters (*Cricetus auratus*) from two different sources were found to present marked differences in their susceptibility to the Lansing strain of poliomyelitis virus. Both strains were found to be markedly inferior to the white mouse and to the cotton rat as experimental animals for poliomyelitis research.

The adjustment of the inoculum at a low pH did not increase the titer of the hamster passage virus consistently. The use of the autolyzed normal mouse brain technic did not increase the infectivity of the virus for the hamster.

The intraperitoneal inoculations of mouse-passage and cotton-rat passage virus resulted in higher titers of neutralizing antibody in the golden hamster than when hamster passage virus was used.

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## 17161. Relation of Pregnancies to Induction of Ovarian Tumors by X-rays.

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Two forces are now postulated in the genesis of ovarian tumors: direct delayed x-ray effect and a hormonal "imbalance." Ova are highly sensitive to x-rays. Their destruction disrupts the normal development of the ovarian follicles. It is believed that new formation of ova occurs in young female mice, therefore, it can be assumed that the hormonal imbalance induced by x-rays in very young mice as well as that caused by small doses of x-rays is slight and under such conditions ovarian tumors are less likely to occur. These were the thoughts that led to the pilot experiments here described.

**Irradiation of Mice at 1-3 Days of Age:** Earlier experiments have shown that a single exposure to 87r or more at 5-10 weeks of age will produce tumors in most mice when they reach adult age.<sup>1</sup>

In the first experiment reported here, mice were irradiated with 150r at 1-3 days of age and were reared by their non-irradiated mothers and allowed to live until natural death or about 20 months of age. At about 4-6 weeks of age they were weaned and a few weeks later mated with normal males. When pregnant they were separated, allowed to nurse their babies after which they were returned to the mating cage. Table Ia indicates that about 30% of these mice became pregnant and some had as many as 4 pregnancies. Ovarian tumors developed in 76%. One-half of those that were pregnant remained free from ovarian tumors as compared to 8% of those that were completely sterilized by x-rays. However, even multiple pregnancies failed to prevent the development of ovarian tumors. The time of appearance of these neoplasms is not significantly shorter than that in mice irradiated at 4-6 weeks of age.<sup>1</sup>

<sup>1</sup> Furth, J., and Boon, M. C., *Cancer Research*. 1947, 7, 241.

TABLE Ia.  
Effect of Irradiation of 1-3-Day-Old Mice (Rf/Ak) with 150r.

Length of life (mo.)	No. in group	No. pregnant	No. of pregnancies				Neoplasms		
			1	2	3	4	Ovary	Lung	Leukemia
7	2	1	1						
8-13	5	2	2				2	1	
14-18	13	6	3	2	1		9	3	3
19-23	39	9	3	2	2	2	34	15	1
Total	59	18	9	4	3	2	45	19	4
%		30.5%					76.3%	32.2%	6.8%

Factors of irradiation were as follows: 140 kv., 5 m. amp., 25 cm target skin distance, with an inherent filtration of 1 mm of aluminum, machine delivering 120r per minute.

TABLE Ib.  
Controls.

Length of life (mo.)	No. in group	Neoplasms		
		Ovary	Lung	Leukemia
7	1	0	0	0
8-13	2	0	0	0
14-18	7	0	1	0
19-23	28	0	9	1
Total	38	0	10	1
%			(26.3%)	(2.6%)

A group of 38 closely related mice were set aside as controls for the incidence of ovarian and other tumors. No pregnancy records were kept on these mice but it may be assumed that they were fertile. None developed ovarian tumors as shown in Table Ib.

While this slight increase in the incidence of leukemia and lung tumors in x-rayed mice may not be significant, the figures are in line with those observed in earlier larger experiments.<sup>2,5</sup>

The brothers of the female mice irradiated at 1-3 days of age with 150r were also observed until natural death or about 20 months of age. Only one developed a testicular interstitial cell tumor.

Ten Ak mice received 150r at the advanced stage of pregnancy. The females born 1 to 5 days after irradiation were au-

topsied at 7 to 15 months of age. None had grossly detectable ovarian tumors. Three examined microscopically have shown changes as those seen in x-rayed animals.

*Irradiation of Mice with 25-200r at 5 Weeks:* In these experiments C3H mice obtained from the L.C. Strong were used. All other stocks of mice tested earlier were highly susceptible to the induction of ovarian tumors and it was thought that the use of C3H mice susceptible to breast cancers might disclose additional facts.

Table II shows that with increase of the irradiating dose the number of pregnancies is reduced while the ovarian tumor incidence is increased. The only figure out of line of this statement is the lower percentage of ovarian tumors in mice irradiated with 100r. Loss from intercurrent disease was high among these mice. The overall low tumor incidence and the above irregularity may be due in part to poor health and early death of many mice.

A summary analysis of all pertinent data is in Table III.

*Comments.* The data here recorded indicate that mice x-rayed at 1-3 days or 5 weeks of age can have one or several normal pregnancies although they are destined to develop ovarian tumors at a later age. The data do not answer the question which group is more liable to develop ovarian tumors, the x-rayed sterile or x-rayed fertile group. Should induction of ovarian tumors be solely due to a hormonal "imbalance" produced by x-rays, one would expect a much lower incidence of ovarian tumors in mice that go through normal

<sup>2</sup> Furth, J., and Furth, O. B., *Am. J. Cancer*, 1936, 28, 54.

<sup>3</sup> Kaplan, H. S., *Cancer Research*, 1947, 7, 141.

<sup>4</sup> Lorenz, E., Heston, W. E., Eschenbrenner, A. B., and Deringer, M. K., *Radiology*, 1947, 49, 274.

<sup>5</sup> Henshaw, P. S., Riley, E. F., and Stapleton, G. E., *Radiology*, 1947, 49, 349.

TABLE II.  
Effect of Irradiation of C3H Mice at 5 Weeks of Age.

Dose	Length of life (mo.)	No. in group	No. of mice pregnant	No. of pregnancies			Neoplasms		
				1	2	3	Ovary	Lung	Leukemia
25r	7	2	2	1	1				
	8-13	7	7	5	2			1	
	14-19	8	4	1	1	2	2	1	1
	Total	17	13	7	4	2	2	3	1
50r	9-13	7	4	2	2				1
	14-19	13	7	7			4	2	
	Total	20	11	9	2		4	2	1
100r	7	1							
	8-13	5	1	1				1	
	14-18	7	1	1			2		
	19-23	3	1	1			1	1	
	Total	16	3	3			3	2	1
200r	9-13	16	3	1			4	1	2
	14-18	5	2	1			2		1
	19-23	5					4	1	
	Total	26	5	2			10	2	3

In addition the following number of mice had borderline or "pretumors"; 6 in the 25r; one in the 50r; and 4 in the 200r groups.

TABLE III.  
Induction of Ovarian Tumors by Small Doses and Relation to Pregnancy.

Mice, stock Age at irradiation: Dose:	C3H						4-6 weeks <sup>†</sup>		Rf/Ak 1-3 days
	25r	50r	100r	200r	25-200r*	50-200r*	87r	175r	150r
% x-rayed developing ovarian tumors	12%	30%	19%	39%	24%	31%	84%	94%	76%
Sterile mice, No.	4	9	13	21	47	43			41
No. developing ovarian tumors	2	2	2	7	13 (27.6%)	11 (25.6%)			37
Pregnant mice, No.	13	11	3	5	32	19			18
No. developing ovarian tumors	0	2	1	3	6 (18.7%)	6 (31.6%)			8

\* Combined.

pregnancies than in those sterilized by x-rays. The immediate hormonal "imbalance" caused by small doses of x-rays is slight if any, yet the liability of these mice to develop ovarian tumors is great. X-rays are powerful mutagens and cause tumors in organs not under hormonal control. It seems that neither the skin nor the hemopoietic tissues wholly recover from even minor damage caused by x-rays.<sup>6</sup> The same seems true for the ovary

and it is possible that a chromosomal change is a basic factor in the genesis of neoplasm induced by x-rays. However subsequent hormonal influences may eventuate or suppress the appearance of ovarian neoplasms.<sup>9</sup>

Data on the factors of induction of ovarian tumors by radiation are meager. It is known that they can be induced by very small doses. The present experiments show that the amount of x-rays required to induce ovarian tumors is about 25 to 50r if given in a single dose. In the experiments of Lorenz and associates<sup>4</sup>

<sup>6</sup> Butterworth, J. S., *Am. J. Cancer*, 1937, **31**, 85.

<sup>7</sup> Earlier experiments cited.<sup>1</sup>

<sup>8</sup> Stone, R. S., *Radiology*, 1947, **49**, 297.

<sup>9</sup> Li, M. H., and Gardner, W. V., *Cancer Research*, 1949, **9**, 35.

a single dose of 50r produced tumors in 70% of the mice. Daily exposure to 0.11r (from radium) with an accumulated total dose of 90r produced ovarian tumors in about 70% of the mice.<sup>4</sup> No hematological changes have been detected with a daily exposure of 0.1r of gamma radiation in animals that subsequently developed ovarian tumors.<sup>10</sup> An increase in the ovarian tumor incidence in mice exposed to single doses of 26n to 90n of fast neutrons is on record.<sup>5</sup> This is probably much above the minimal tumor producing dose. If mutation is the basis of tumor induction by x-rays, with an increase in dosage an increase in the number of tumors should be anticipated. The present experiments do not exclude this possibility in the range of about 25 to 200r while earlier experiments in the dose range above 87r<sup>1</sup> and those of Lorenz and associates<sup>4</sup> failed to show such a trend.

The relation of age to tumor induction was studied by Kaplan<sup>3</sup> who irradiated 2 weeks to 6 months old mice with 600r administered in daily doses of 50r on 12 consecutive days. The greatest ovarian tumor incidence occurred in mice irradiated at 1 month. None was noted in 7 mice irradiated at 2 weeks but intercurrent mortality was high in this group. The present studies show that mice are susceptible even at 1-3 days of age.

A follow-up of these pilot experiments is desirable, even though each requires about 3 years. The relation of the dose of x-rays and age of hosts to the rate of ovarian tumor in-

duction requires a closer check. The hormonal state of mice preceding tumor development and the incidence of pregnancies and abortions should be determined more accurately. The histological changes in ovaries following irradiation ante- and postpartum deserve further consideration; particularly the alleged post-natal ovogenesis and the regenerative phase which leads to neoplasms. Does irradiation induce a chromosomal change and what initiates and sustains the hormonal imbalance are questions to be answered.

**Summary.** Mice exposed to 50-200r of x-rays can have one or several normal pregnancies and still be liable to the development of ovarian tumors in a stock in which this neoplasm is practically non-existent.

Irradiation at 1-3 days of life with 150r sterilized only 1/3 of the mice while ovarian neoplasms appeared in about 76%. Irradiation at 1-3 days did not hasten the onset of ovarian neoplasms as compared to irradiation at 4-10 weeks. In both groups the tumors developed in middle aged and old mice. The growth rate of the tumors was likewise slow, hardly interfering with the normal life span of these animals.

It is postulated that a specific delayed x-ray effect coupled with a hormonal imbalance provoked by x-rays leads to the development of ovarian tumors.

These experiments were begun at Cornell University Medical College and assisted by Thelma Weaver Mold.

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<sup>10</sup> Jacobson, L. O., and Marks, E. K., *Radiology*, 1947, 49, 286.

## 17162. Correction of Steatorrhea in Bile Fistula Dogs by Frequent Return of Bile.\*

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It was previously reported that 3 to 6 g daily doses of various bile acid preparations mixed with a fatty meal were at best only

partially effective in correcting the steatorrhea of bile fistula dogs.<sup>1</sup> This was attributed to one or more of the following possibilities: a,

\* Supported in part by a grant from G. D. Searle & Co., Chicago, Ill.

<sup>1</sup> Heersma, J. R., and Annegers, J. H., *Proc. Soc. Exp. Biol. and Med.*, 1945, 67, 339.

a physiologically inactive bile preparation; *b*, an inadequate total dosage; *c*, improper time of administration. In subsequent studies, *a* and *b* were minimized by returning all of animals' own bile to the intestine instead of giving commercial bile preparations. As to *c*, flow of concentrated gallbladder bile into the intestine during gastric evacuation and digestion of a fatty meal was shown to be unnecessary for normal fat absorption, since no steatorrhea developed in cholecystectomized dogs.<sup>2</sup> The present experiments involving the return of bile fistula dogs' bile at different intervals throughout the day were designed to determine whether bile must be present continuously for normal fat absorption.

**Methods.** Rous-McMaster external bile fistula dogs<sup>3</sup> were prepared with an additional rubber tube for return of bile into the duodenum.<sup>4</sup> Beginning 7 to 10 days after operation, diets containing 335 g of commercial dog food ("Pard") and 25 g of lard (36 g of total fat) were fed once daily. All meals were completely consumed. Total fecal fat excretion was determined<sup>5</sup> on 5-day stool collections when no bile was returned and when the animals' own bile was returned. Cholic acid in the bile returned during 5-day periods was determined<sup>6</sup> on pooled aliquots. The bile collected during 8, 4, or 1-hour intervals was introduced into the duodenum at the rate of 5 cc per minute.

**Results.** When no bile was returned, fecal fat excretion averaged 25.7 g per day in 6 dogs. This value is in agreement with fecal fat excretion of 27.3 g per day obtained in a previous study in 9 cholecystonephrostomized dogs receiving the same diet.<sup>7</sup>

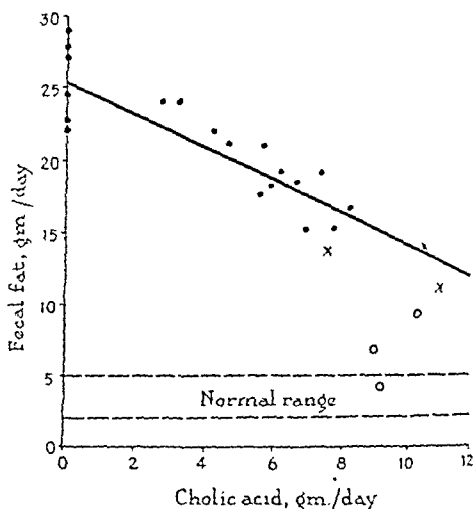


FIG. 1.

Relationship between fecal fat excretion and cholic acid of whole dog bile introduced into the intestine.

● Bile returned every 8 hr  
 × " " " 4 " "  
 ○ " " " 1 "

The straight line represents the regression equation fitted to the filled circles only.

In 4 dogs bile was returned every 8 hours for 2 to 10 consecutive 5-day periods. The daily quantity of bile acid secreted and returned increased to reach a steady-state level by the third period, in agreement with the results of Berman *et al.*<sup>4</sup> The average daily quantity of cholic acid secreted and returned during the 5-day tests, and the corresponding daily excretion of fecal fat are plotted for each dog in Fig. 1 (filled circles). As the dose of cholic acid increased, the fecal fat output decreased in linear fashion; the regression equation (correlation coefficient was  $-0.88$ ) indicated that for each gram increase in cholic acid return fecal fat excretion decreased by 1.17 g. However, at the steady-state level of cholic acid secretion and return (6 to 8 g/day), the defect in the fat absorption was only about 50% corrected. Extrapolation of the regression line implied that 20 g of cholic acid per day would be required for normal fat absorption when the bile is returned every 8 hours. This amount greatly exceeds the steady-state output obtained in this and a previous study,<sup>4</sup> and hence indicates that a time factor as well as a dose factor must be concerned.

<sup>2</sup> Heersma, J. R., and Annegers, J. H., *Proc. Soc. Exp. Biol. and Med.*, 1948, **69**, 140.

<sup>3</sup> Rous, P., and McMaster, P. D., *J. Exp. Med.*, 1923, **37**, 11.

<sup>4</sup> Berman, A. L., Snapp, E., Ivy, A. C., and Atkinson, A. J., *Am. J. Physiol.*, 1941, **131**, 776.

<sup>5</sup> Fowweather, F. S., and Anderson, W. N., *Biochem. J.*, 1946, **40**, 350.

<sup>6</sup> Irvin, J. L., Johnson, C. G., and Kopola, J., *J. Biol. Chem.*, 1944, **153**, 439.

<sup>7</sup> Heersma, J. R., and Annegers, J. H., *Am. J. Physiol.*, 1948, **153**, 143.

Accordingly, bile was returned every hour in 3 dogs for 6 days. Fat absorption during the last 5 days was within normal limits<sup>7</sup> in one dog and considerably improved in the others. Since the plotted points (open circles) are well below the regression line in Fig. 1, the improvement appeared to be due largely to the continuous presence of bile, and not to the increased quantity of cholic acid secreted and returned. To check this point further, bile was returned every 4 hours in the same 3 dogs. The quantity of cholic acid returned did not change, but fecal fat excretion increased so that the plotted values (crosses) fell near the original regression line.

Finally, fecal fat excretion was measured in 3 dogs after cessation of bile return, in order to determine the rapidity with which the fat absorptive defect develops when bile is excluded. From the third through the seventh day after bile return was stopped the fecal fat excretion averaged 17.8 g per day; from the eighth through the thirteenth day, 22.0 g per day. The former value is significantly less, by group comparison analysis,<sup>8</sup> than the mean fecal fat excretion obtained in the present and previous<sup>7</sup> study during 5-day periods begun 10 days or more after the operation. The results indicate that impaired fat absorption develops rapidly when the bile is first excluded, then more slowly to a maximum after about 7 days. The presence of bile acids in the scraped intestinal mucosa of dogs 24 hours, but not 1 month after bile exclusion,<sup>9</sup> suggests a possible reason for the de-

lay in development noted.

**Discussion.** The present results demonstrate that previous failure to correct steatorrhea in bile fistula dogs by feeding them whole ox-bile containing 6 g of cholic acid with the single daily meal was probably due to the failure of this procedure to provide adequate amounts of the bile in the intestine over the period required to complete fat absorption. Although continuous hourly administration of bile was employed successfully in this study to restore normal fat absorption, this does not imply that hourly return must be continued *throughout a 24-hour day* to complete the absorption of a single daily fat meal. However, the establishment of the fact that bile must be present in the intestine continuously for at least most of the day now makes it possible to investigate further the bile component(s) and dosages necessary for normal fat absorption, and to assay bile replacement measures.

**Summary.** The steatorrhea of 3 bile fistula dogs given a fatty meal once daily was nearly completely corrected when the animals' own bile was returned to the duodenum every hour. The steatorrhea persisted, however, when bile was returned every 4 or 8 hours. Since the total quantity of cholic acid returned during the 1- and 4-hour regimes was the same, the importance of continuous presence, apart from total daily quantity present, in the intestine is demonstrated.

<sup>9</sup> Verzar, F., and Von Kuthy, A., *Biochem. Z.*, 1931, **230**, 451.

<sup>8</sup> Snedecor, G. W., *Statistical Methods*, Iowa State College Press, 1946.

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## 17163. Observations on Experimental Dental Caries. XI. Influence of Pregnancy.

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The results of the clinical investigations on the relationship between pregnancy and dental caries have been contradictory. However, most of the controlled studies made in the last years negate the validity of the old popu-

lar idea that pregnancy increases the susceptibility to dental caries. Day,<sup>1</sup> for example,

<sup>1</sup> Day, C. D. M., *Indian J. Med. Research*, 1947, **35**, 101.



in a recent study carried out with a large number of subjects who had gone through various numbers of pregnancies, and who were divided into several age groups, found no significant differences which could support the statement of a relationship between pregnancy and dental caries incidence. Furthermore, Ziskin and Hotelling,<sup>2</sup> also in clinical studies, found evidence to show that pregnancy not only does not increase dental caries but that factors operating during pregnancy actually prevent tooth decay to a significant degree.

In hamsters, we<sup>3</sup> found that pregnancy and lactation do not influence caries activity in any direction. Considering Ziskin and Hotelling's findings, we have studied in the present experiment the influence of pregnancy alone, in order to test the possibility that a significant decrease of dental caries activity brought about by pregnancy alone, might be annulled by a harmful effect of lactation.

**Experimental.** Thirty female hamsters, 23 days old, from litters of a colony maintained on Purina Laboratory Chow\* and raw milk, were littermate distributed into two groups as follows: Group I, 17 females, and Group II, 13 females. Both groups were reared for 100 days on the following diet of sub-optimal nutritional value: finely ground yellow corn 25%, sucrose 25%, corn starch 20%, powdered whole milk 22%, ether-extracted yeast 5%, alfalfa meal 2%, salt mixture† 1%, and d,l- $\alpha$ -tocopherol acetate 0.010%. The ration and tap water were available *ad libitum*.

Both groups were kept during the first 56 experimental days in screen bottom cages without bedding. At this time the 2 groups were transferred to breeding cages containing sawdust on the bottom, and excelsior for bedding; thereafter 9 sexually mature males kept on the stock colony diet were mated for 13 days with the 17 females of Group I.

<sup>2</sup> Ziskin, D. E., and Hotelling, H., *J. D. Res.*, 1937, **16**, 507.

<sup>3</sup> Granados, H., Glavind, J., and Dam, H., *Odontologisk Tidskrift*, 1948, **56**, 388.

\* From Ralston Purina Co., St. Louis, Mo.

† The salt mixture used was McCollum's Salt Mixture No. 185, supplemented with 13.5 mg KI, 139 mg CuSO<sub>4</sub>, 5H<sub>2</sub>O, and 556 mg MnSO<sub>4</sub>, 4H<sub>2</sub>O per 100 g.

TABLE I.  
Caries Activity in the Two Groups.

	Group I (pregnant) 14 ♀ ♀	Group II (nonpregnant) 13 ♀ ♀
% of animals affected	100	100
Avg No. of carious molars	8.0	8.5
Avg No. of carious lesions	11.5	10.8
Avg caries scores	6.0	6.2

Every litterborn from Group I was removed from the mother within 6 hours after parturition. Both groups remained in the breeding cages until the end of the experiment.

On completion of the 100 day experimental period the animals were sacrificed and autopsied. The molars were prepared for examination in the usual way,<sup>4</sup> and the carious lesions were recorded and scored.<sup>5</sup>

**Results.** In group I out of the 17 mated females 2 died during labor, and one had sterile mating. Therefore, only 14 females, those who had normal parturitions, finely formed Group I.

Table I shows the caries activity of the two groups. It is apparent that essentially the same incidence and extent of carious lesions were found in both groups. Therefore, under the conditions of this experiment, pregnancy did not influence in any direction the caries activity. Thus can be stated that neither pregnancy and lactation,<sup>3</sup> nor pregnancy alone influence in any direction the caries susceptibility of the Syrian hamster. These experimental findings agree with the results of most of the controlled clinical studies carried out in the last years<sup>1</sup> on this subject.

It is generally recognized that pregnancy and lactation are physiological states of increased nutritional stress for the mother, since she has to face the double task of supplying proper amounts of nutrients necessary for the offspring as well as her own metabolism. Certain workers<sup>6</sup> have stated that the increased caries activity during pregnancy reported by them has been due to

<sup>4</sup> Granados, H., Glavind, J., and Dam, H., *Acta Pathol. et Microbiol. Scandinav.*, 1948, **25**, 453.

<sup>5</sup> Keyes, P. H., *J. D. Res.*, 1944, **23**, 439.

<sup>6</sup> Wohl, M. G., Dietotherapy. Clinical application of modern nutrition, W. B. Saunders Co., Philadelphia, 1945, p. 540.

quantitative and/or qualitative deficiencies in the diet. In our previous experiment on the influence of pregnancy and lactation<sup>2</sup> on caries activity, as well as in the present study, we used a sub-optimal diet in order to sharpen the nutritional deficiency of the group undergoing pregnancy, and thus obtain sharper differences of caries activity between the two groups in case of any relationship between dietary deficiencies and dental caries.

However, as it can be seen from the data presented, neither pregnancy and lactation<sup>3</sup> nor pregnancy alone influenced caries activity

in any direction. These facts are in open contradiction with the alleged increase of caries activity as a consequence of dietary deficiencies during pregnancy.

**Summary.** The influence of pregnancy on caries activity has been studied in hamsters reared from weaning for 100 days on a sub-optimal diet, with their respective littermate controls. The results show that pregnancy does not influence caries activity in any direction.

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# 17164. Evidence for a Steroid Compound in Cane Juice Possessing Antistiffness Activity.

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The isolation from crude cane molasses and crude unheated cane juice of a fraction capable of alleviating an induced wrist stiffness in guinea pigs raised on a skim milk diet, was described by van Wagtendonk and Wulzen.<sup>1</sup> Later investigations by Oleson, *et al.*,<sup>2</sup> and Petering and coworkers<sup>3</sup> indicate that ergostanyl acetate and  $\alpha$ -ergostanyl acetate are effective curative agents. We are therefore prompted to report that an active fraction of steroid nature has been isolated from the original cane juice fraction, and in our assay methods has proved to be a curative agent in dosages of at least 0.01  $\gamma$ .

**Experimental.** A product obtained from the cane juice and melting at 81-82°<sup>1</sup> did not prove to be homogeneous when subjected to carbon-hydrogen analyses. It was therefore subjected to various procedures for further

purification. Chromatic adsorption on columns of magnesium oxide or activated alumina failed to yield pure and homogeneous products. Repeated recrystallization from alcohol, followed by recrystallizations from acetone and cyclohexane yielded a compound melting at 164-166° that was homogeneous according to the carbon and hydrogen analyses and highly active in curing the induced stiffness in deficient guinea pigs.

**a. Activity tests.** The assay for activity was carried out according to the previously described methods.<sup>1,4</sup> The compound was dissolved in Wesson oil and administered orally to guinea pigs that had been kept on the deficient regime of skim milk powder to which the necessary vitamins and minerals had

TABLE I.

Therapeutic Test of High Melting Compound Carried Out on Guinea Pigs on Skim Milk Ration.

Level of assay in $\gamma$ (5 times)	No. of animals	Cured	No cure
1	3	3	0
0.01	27	23	4

\* Present address: Argonne National Laboratory, Chicago, Ill.

<sup>1</sup> van Wagtendonk, W. J., and Wulzen, R., *J. Biol. Chem.*, 1946, **164**, 597.

<sup>2</sup> Oleson, J. J., van Donk, E. C., Bernstein, S., Dorfman, L., and Subbarow, Y., *J. Biol. Chem.*, 1947 **171**, 1.

<sup>3</sup> Petering, H. G., Stubberfield, L., and Delor, R. A., *Arch. Biochem.*, 1948, **18**, 487.

<sup>4</sup> van Wagtendonk, W. J., and Wulzen, E., *Arch. Biochem.*, 1943, **1**, 373.

TABLE II.  
 Carbon and Hydrogen Analyses of the High Melting Compound.

Compound	C	H	Calculated for:					
			$C_{27}H_{44}O$		$C_{28}H_{46}O$		$C_{30}H_{50}O$	
	C	H	C	H	C	H	C	H
High melting	84.35	11.73*						
	84.50	11.61*						
	83.43	11.38†						
High melting recovered from digitonin ppt.								
	84.02	11.55*						
Avg	84.06	11.57	84.31	11.53	84.35	11.63	84.44	11.81

\* Analyses by Dr. A. J. Haagen Smit, California Institute of Technology.

† Analyses by Dr. H. L. Hunter, Eli Lilly and Company.

been added.<sup>5</sup> The results of the assay of the high melting compound (164-166°) are reported in Table I.

*b. Chemical properties.* The high melting compound gave a strong positive Liebermann-Burchard reaction. The compound could be precipitated with digitonin, and the original compound could be recovered from the digitonin precipitate by digestion with pyridine. The compound and its acetate showed an ultraviolet absorption spectrum (Fig. 1) with an inflection at 262-265 mμ, and maxima at 272, 283, and 295.5 mμ. The values of the extinction coefficient at the maxima were, for the high melting compound, respectively, 74.5, 77.1, and 45.5, and, for the acetate, respectively 35.6, 36.9, and 21.0. The carbon and hydrogen analyses of the high melting compound and of the product reisolated from the digitonin precipitation are given in Table II.

The acetate was prepared by refluxing 200 mg of the high melting compound for 30 minutes with 10 ml of acetic anhydride. The mixture was cooled and filtered. The crystals were washed, dried and recrystallized twice from 95% ethanol. The yield was 185 mg

of colorless leaflets, melting at 141-143°, and having the following analysis:

Found:	C: 82.55	H: 11.02
	82.67	11.48
	81.63	11.03
	Avg	82.35 11.18
Calculated for:	$C_{30}H_{48}O_2$	81.76 10.98
	$C_{32}H_{52}O_2$	81.94 11.18

The acetate was saponified according to Sandquist and Gorton<sup>6</sup> with the results shown in Table III. The high melting compound was hydrogenated with platinum oxide as catalyst and acetic acid as solvent with the results reported in Table IV.

 TABLE III.  
 Equivalent Weight Determination of the High Melting Compound.

Weight sample, mg	ml HCl, 0.100 N	Equivalent weight
46.5	1.10	447.2
63.3	1.39	455.4
55.2	1.24	445.2
Avg		449.3
Calculated for:	$C_{26}H_{46}O_2$	426.6
	$C_{30}H_{48}O_2$	440.69
	$C_{32}H_{52}O_2$	468.74

 TABLE IV.  
 Hydrogenation of High Melting Compound.

Compound	Catalyst, mg	Sample, mg	H <sub>2</sub> taken up, ml	Time of hydr.	Moles of H per mole compound
Cholesterol high melting compound	3.025	11.711	0.684	30 min.	1.00
	3.011	10.988	1.190	25 min.	1.93
	3.268	11.887	1.301	44 min.	1.89
	2.878	14.872	1.629	10 hr	1.93

<sup>5</sup> van Wageningen, D. J., and Wulzen, R., *Arch.* 155, 337.

<sup>6</sup> Sandquist, H., and Gorton, J., *Ber.* 1930. 63B. 1935.

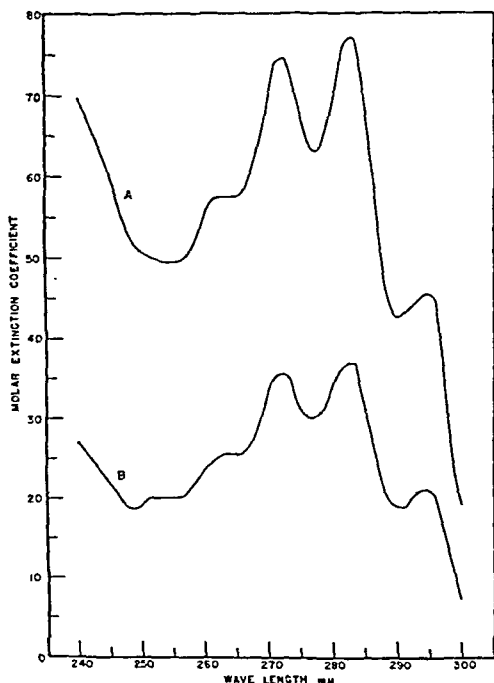


FIG. 1.

Ultraviolet adsorption of the high melting compound (curve A) and its acetate (curve B).

The acetate was hydrogenated in the same manner. The product of the hydrogenation was recovered from the solution and recrystallized once from 95% ethanol. The compound melted at 130.5-132.0° and had the following composition:

Found:	C: 80.52	H: 12.02
	80.71	12.03
	Avg	80.62 12.02
Calculated for: $C_{29}H_{50}O_2$	80.87	11.70
$C_{30}H_{52}O_2$	81.05	11.80
$C_{32}H_{56}O_2$	81.29	11.94

**Summary.** A compound of steroid nature, having the probable formula of  $C_{28}H_{46}O$ , has been isolated from cane juice. This compound has therapeutic activity in alleviating an induced stiffness in guinea pigs. It has not been possible to further elucidate the structure of this compound, since not enough material was available at the time the project had to be discontinued, due to circumstances beyond control of the two senior authors. The isolations, however, tend to confirm the findings by Oleson *et al.* and Petering *et al.*, in that the antistiffness factor is steroid in nature.

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### 17165. Electron Microscope Studies of the Vesicle and Spinal Fluids from a Case of Herpes Zoster.\*

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The pathogenesis of herpes zoster has not been resolved. Even though recent work has suggested that the virus may multiply in the skin, the part played by the central nervous system in this infection is not clear. Like the virus of varicella, with which it is either closely related or identical, the virus of herpes

zoster cannot readily be passed to laboratory animals, making work difficult in this disease. However, elementary bodies of varicella and herpes zoster have been described as occurring in vesicular fluids when such fluids have been examined in the optical microscope.<sup>1-3</sup> These bodies have been characterized more fully in the electron microscope by Ruska,<sup>4</sup> by Rake

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<sup>†</sup> National Institutes of Health Postdoctorate Research Fellow.

Appreciation is expressed to Dr. John Craig for aid in obtaining specimens.

<sup>1</sup> Paschen, E., *Hyg. Rundschau*, 1919, **29**, 269, 313.

<sup>2</sup> Aimes, C. R., *Lancet*, 1933, **224**, 1015.

<sup>3</sup> van Rooyen, C. E., and Illingsworth, R. S., *Brit. Med. J.*, 1944, **2**, 526.

<sup>4</sup> Ruska, H., *Scientia*, 1943, **37**, 16.

and his coworkers,<sup>5,6</sup> and by Farrant and O'Connor.<sup>7</sup> We have been able to confirm these findings in regard to finding elementary bodies in the vesicular fluids of varicella and of herpes zoster, and in addition have demonstrated particles of similar size in the cerebrospinal fluid of a patient with herpes zoster. The purpose of this paper is to present these findings and to compare the particles with those found in the vesicular fluid from a case of herpes simplex.

**Case report.** The patient studied was a 28-year-old post-graduate student who was admitted to the New Haven Hospital on December 14, 1948 with complaints of frontal headache of 10 days duration becoming more severe in the last 5 days, and of fever of 3 days duration. He had not noted chest pain or a skin rash. Previous hospital records disclosed that he had been under investigation for an unexplained splenomegaly and hyperglobulinemia since 1944. Physical examination, including a neurological evaluation, was not remarkable except for a group of early vesicles on an erythematous base which extended from the 9th and 10th thoracic vertebra posteriorly nearly to the mid-line anteriorly on the left side over the 6th and 7th ribs, and a spleen that was palpable 2 cm below the costal margin. There was no hyperaesthesia over the affected skin areas. Laboratory work on the day of admission revealed a normal white, red, and differential count. A lumbar puncture done on the following day yielded clear fluid with the following characteristics: leucocyte count 145 per cmm, all of which were mononuclear, protein 156 mg%, Wassermann negative and bacterial culture negative. Initial pressure was 170 mm of water, final pressure, 120. A second spinal puncture on the next day revealed an increase in cell count to 225, which were predominantly lymphocytes. The patient remained asymptomatic

except for headache, but had daily fever up to 102°F for 4 days. Convalescence was uneventful.

**Materials and methods.** For the electron microscope preparations, vesicle fluid from the patient was obtained with a tuberculin syringe on the day following admission. It was collected in 0.5 ml of saline, and after a clearing centrifugation at 2000 r.p.m. for 20 minutes was spun at 18,000 r.p.m. for 30 minutes in a cooled rotor, 6 inches in diameter. The sediment was resuspended in 0.2 ml of distilled water. Two samples of cerebrospinal fluid of this patient in amounts of 2 and 8 ml were handled in a similar fashion as were the samples of vesicular fluid from cases of varicella. The specimen of herpes simplex fluid shown was obtained from a lesion on the upper lip of a patient with a respiratory infection. It was collected in 0.2 ml of saline and examined without centrifugation. Spinal fluid from 20 patients with various ailments (tuberculous meningitis, poliomyelitis, and noninfectious neurological disorders) were examined, 15 without centrifugation and 5 after centrifugation as above.

Small drops of the samples were placed on collodion covered 200-mesh stainless steel grids and allowed to dry. They were then shadow-cast with chromium at an angle of 1 to 7, and examined in a RCA electron microscope, type EMU. The magnification was calibrated by photographing a silica replica of a diffraction grating (15,000 lines per inch) and by the

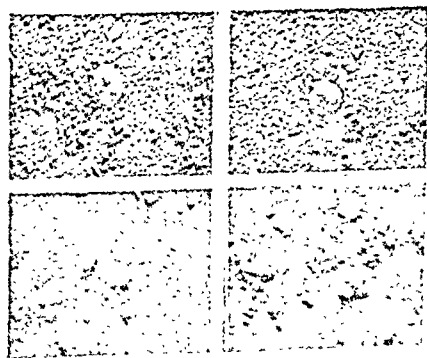


FIG. 1.  
Electron micrographs of vesicle fluid from a patient with herpes zoster.  $\times 20,700$ .

<sup>5</sup> Nagler, F. P. O., and Rake, G., *J. Bact.*, 1948, 55, 45.

<sup>6</sup> Rake, G., Blank, H., Coriell, L. L., Nagler, F. P. O., and Scott, T. F. M., *J. Bact.*, 1948, 56, 293.

<sup>7</sup> Farrant, J. L., and O'Connor, J. L., *Nature*, 1949, 163, 260.

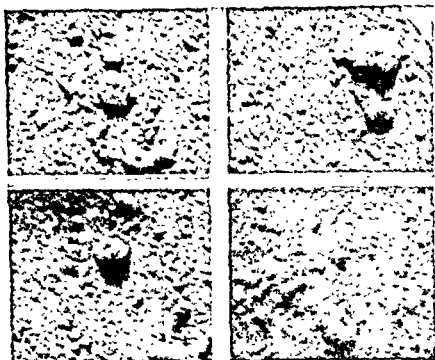


FIG. 2.

Electron micrographs of spinal fluid from a patient with herpes zoster.  $\times 20,700$ .

use of latex balls of 259 m $\mu$  diameter as described by Williams and Backus.<sup>8</sup>

**Results.** Fig. 1 illustrates typical bodies found in the vesicular fluid of the patient with herpes zoster and Fig. 2 those found in his spinal fluid. As seen from the frequency dis-

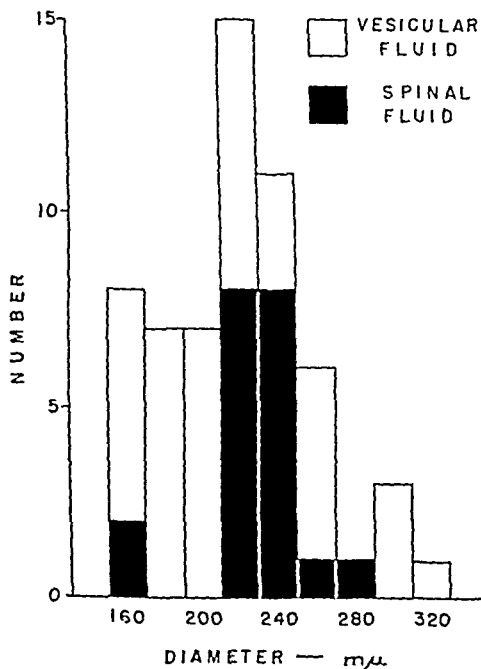


FIG. 3.

Frequency distribution of particles seen in the vesicular and spinal fluids of a case of herpes zoster.

<sup>8</sup> Williams, R. C. and Backus, R. C., *J. App. Phys.*, 1948, 19, 119.

tribution of particles of varying diameters in Fig. 3, there is no significant difference between the average diameter of the elementary bodies found in either fluid, being 230 m $\mu$  for those in the vesicular fluid and 224 m $\mu$  for those in the spinal fluid.

No bodies of this sort were seen in any of the spinal fluids examined from patients with other diseases.

Fig. 4 illustrates elementary bodies obtained from vesicles of a patient with chicken pox, the average diameters of the particles being 244 m $\mu$  (range 200-280). The diameters of particles from 2 other patients with varicella averaged 250 m $\mu$  (range 190-320) and 225 m $\mu$  (range 180-250). The overall average of the particles from these 3 patients was 245 m $\mu$ .

Fig. 5 is a micrograph of elementary bodies from vesicle fluid of a patient with herpes simplex showing elementary bodies having an average diameter of 213 m $\mu$  (range 200-220).

It is difficult from any of our photographs to speak of brick-shaped elementary bodies such as discussed by Rake *et al.*<sup>5</sup> However some of the particles appear to have corners and in the measurements the average diameter of each particle was used. The preparations seem to be characterized by surface irregularities and particularly a central area of depression in the middle of which often appears a small elevated round structure. The latter was particularly marked in the bodies from herpes simplex (Fig. 5).

**Discussion.** These observations tend to confirm those recently presented by Rake and his colleagues<sup>6</sup> as well as those of Farrant and O'Connor<sup>7</sup> bearing on the similarities in elementary bodies observed in vesicular fluids from patients with herpes zoster and with varicella. Moreover, in addition to finding elementary bodies in the vesicular fluid of a patient with herpes zoster we have found particles of similar size in the spinal fluid of this patient. It is noteworthy that in the absence of clinical evidence of central nervous system involvement other than headache this patient showed a pleocytosis of 145 and 225 cells (chiefly lymphocytes) per cmm on 2 successive days during his disease. In a series

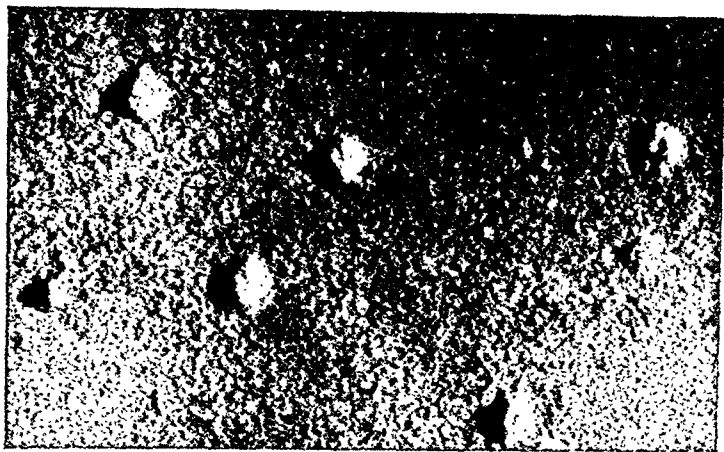


FIG. 4.

Electron micrographs of vesicle fluid from a patient with chicken pox.  $\times 36,200$ .

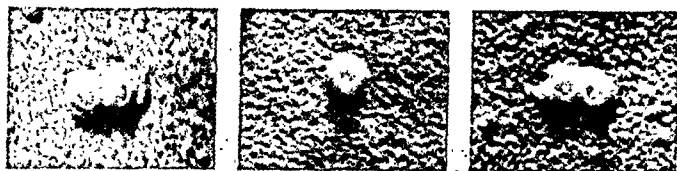


FIG. 5.

Electron micrographs of vesicle fluid from a patient with herpes simplex.  $\times 27,500$ .

of 137 cases of herpes zoster reported by Gais and Abrahamson<sup>9</sup> lumbar punctures were done in 11: in 6 the count was normal while 5 showed a pleocytosis of 40 to 110 cells per cmm; chiefly lymphocytes.

Whereas herpes simplex appears to be unrelated to herpes zoster, similar appearing bodies have been detected in vesicular fluid here also. These bodies are 200-220  $\mu$  in diameter which is considerably larger than that found by filtration studies of herpes virus contained in rabbit testicle and brain tissue by Elford *et al.*<sup>10</sup> However to our knowledge,

filtration studies of the virus in vesicular fluid have not been done.

*Summary.* 1. Electron microscope studies of vesicular and spinal fluids from a patient with herpes zoster have demonstrated in both particles of similar size averaging 227  $\mu$  in diameter.

2. These particles are compared to those in vesicular fluids from patients with herpes simplex and chicken pox. In herpes simplex particles averaging 213  $\mu$  in diameter were found, and in chicken pox, particles of 245  $\mu$ .

<sup>10</sup> Elford, W. J., Perdrau, J. R., and Smith, W., *J. Path. and Bact.*, 1933, **30**, 49.

<sup>9</sup> Gais, E. S., and Abrahamson, R. H., *Am. J. Med. Sci.*, 1939, **197**, 817.

# 17166. Nature of the Plasma Factor Responsible for *In vitro* Lysis of Leucocytes by Tuberculo-protein.\*

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*In vitro* tissue culture studies on the tuberculin reaction,<sup>1</sup> using washed buffy coats of sensitized animals, have stressed the role of cells rather than serum antibodies in the tuberculin type of hypersensitivity. Since the tuberculin reaction may be passively transferred to a normal host with white cells and not with the serum from sensitized animals,<sup>2</sup> and since white cell suspensions from tuberculous animals<sup>3</sup> and humans<sup>4</sup> are lysed by tuberculin within one hour, it would seem that the delayed type of hypersensitivity is in some way mediated via the white cells of the animal body.

In an earlier report<sup>5</sup> using the one hour observation technic on white cell suspensions it was noted that white cells of normal tuberculin-negative and tuberculous humans have an equal affinity for tuberculin antigen. Subsequent experiments<sup>6</sup> with the same technic, using thoroughly washed white cells of both normal tuberculin-negative humans and tuberculous patients, indicate that the one hour *in vitro* cytotoxic effect of tuberculin is dependent on the presence of some factor in tuberculous plasma. The present report extends

these observations on the tuberculous plasma factor.

*Experimental.* Because normal white blood cells were shown to undergo the same lysis as do sensitized cells when incubated with tuberculin antigen and tuberculous plasma<sup>6</sup> and in order to eliminate any property of the tuberculous cell as a factor in cytolysis, thoroughly washed white cells from healthy tuberculin-negative human subjects were used in all experiments. Tuberculous plasma (or serum) was obtained from tuberculous patients hospitalized for acute tuberculosis. Normal white cell concentrates were diluted with this plasma in amounts sufficient to bring the final cell concentration to the range of 4,000-15,000 cells per cu mm. The tuberculous plasma was used in several ways:

- a) freshly drawn;
- b) after storage at 10°C for 7 days;
- c) after dialysis for 2 days against isotonic saline;
- d) after heating at 56°C for 15 minutes; and
- e) as two fractions obtained as follows:
  1. the globulin precipitated by dialysis of the plasma for 2 days against distilled water; and

2. the supernatant plasma freed of precipitate by centrifugation and redialized against isotonic saline. The supernatant plasma was added to one tube containing normal cells; the precipitate, after being suspended in 1 ml of isotonic saline, was added to a similar tube of normal cells.

In all cases where tuberculous plasma was subjected to heat or was used at any time subsequent to the day of collection from the patients, fresh normal guinea pig serum, containing approximately 400 units of active complement per ml, was added to the medium. To 0.4 ml of such white cell suspensions was

\* Work done under an U.S.P.H.S. Research Grant.

<sup>†</sup> Research Fellow, National Institute of Health.

<sup>1</sup> Rich, A. R., and Lewis, M. R., *Bull. Johns Hopkins Hosp.*, 1932, **50**, 115.

<sup>2</sup> Chase, M., *Proc. Soc. Exp. Biol. and Med.*, 1945, **59**, 134.

<sup>3</sup> Favour, C. B., *Proc. Soc. Exp. Biol. and Med.*, 1947, **65**, 269.

<sup>4</sup> Fremont-Smith, P., and Favour, C. B., *Proc. Soc. Exp. Biol. and Med.*, 1948, **67**, 502.

<sup>5</sup> Favour, C. B., *Proc. Soc. Exp. Biol. and Med.*, 1949, **70**, 369.

<sup>6</sup> Miller, J. M., Favour, C. B., Wilson, B. A., and Umbarger, M. A., *Proc. Soc. Exp. Biol. and Med.*, 1949, **70**, 738.



TABLE I.

*In vitro* Effect of Tuberculin on Normal Leucocytes in Presence of Normal and Tuberculous Plasma Before and After Saline Dialysis at 10°C.

Normal cells	X	X	X	X	X	X	X	X
Tbc. Plasma (freshly drawn)	X	X						
Normal Plasma (freshly drawn)			X	X				
Tbc. Plasma (dialysis 48 hr against isotonic saline)					X	X		
Normal Plasma (dialysis 48 hr against isotonic saline)							X	X
Tuberculin antigen	0.1		0.1		0.1		0.1	
Saline		0.1		0.1		0.1		0.1
Total WBC								
5 min.	8,770	9,420	8,530	9,450	7,380	8,290	9,270	11,260
60 min.	6,510	9,390	8,500	9,360	5,330	8,320	9,210	11,080
% Decrement	-25.8	-0.3	-0.5	-0.8	-27.6	+0.2	-0.7	-1.5

added 0.1 cc of tuberculin antigen (prepared as described earlier<sup>5</sup>). Cell system controls with isotonic saline replacing the tuberculin antigen were studied in similar manner. In every instance, normal plasma from a tuberculin-negative donor was subjected to the same treatment as was tuberculous plasma (heat, dialysis, etc.) and added to normal white cell concentrates in the presence of "Old Tuberculin" and saline. Cytolysis was demonstrated by doing total white counts before and after a 60 minute period of incubation at 37°C. The accompanying protocols (Tables I, II, III) illustrate the method of experimentation. Essentially similar results have been obtained many times on different tuberculous patients.

**Results.** 1. Washed white cells from normal tuberculin-negative humans undergo significant cytolysis when suspended in the plasma of tuberculous patients in the presence of "Old Tuberculin" under conditions of these experiments (Table I).

2. The ability of tuberculous plasma to produce cell lysis is retained after dialyzing such plasma against isotonic saline for 48 hours at 10°C (Table I).

3. Heating tuberculous plasma to 56°C for 15 minutes destroys its ability to lyse white

TABLE II.

*In vitro* Effect of Tuberculin on Normal Leucocytes in Presence of Tuberculous Plasma Before and After Heating Plasma to 56°C for 15 Minutes.

Normal cells	X	X	X	X
Tbc. plasma	X	X		
Tbc plasma* (heated 15 min. at 56°C)			X	X
Tuberculin antigen	0.1		0.1	
Saline		0.1		0.1
Total WBC				
5 min.	7,790	7,470	7,310	6,730
60 min.	5,640	7,540	7,270	6,720
% decrement	-24.8	+0.8	-0.8	-0.3

\* Fresh guinea pig complement added.

cells, even when fresh guinea pig complement is added to the system (Table II).

4. If tuberculous plasma is dialyzed at 10°C for 48 hours against distilled water, a white precipitate appears. The supernatant plasma, after redialysis against isotonic saline, has no effect on white cells in the presence of "Old Tuberculin." The precipitate, when resuspended in isotonic saline, is capable of causing white cell cytolysis (Table III).

TABLE III.  
*In vitro* Effect of Tuberculin on Normal Leucocytes in Presence of Normal and Tuberculous Plasmas  
 Before and After Dialysis Against Distilled Water.

Normal cells	X	X	X	X	X	X	X	X	X	X	X	X
Tbc. plasma*	X	X										
Normal plasma*			X	X								
Supernatant of tbc. plasma					X	X						
Supernatant of normal plasma							X	X				
Globulin precipi- tate tbc. plasma									X	X		
Globulin pre- cipitate normal plasma											X	X
Tuberculin antigen	0.1		0.1		0.1		0.1		0.1		0.1	
Saline		0.1		0.1		0.1		0.1		0.1		0.1
Total WBC												
5 min.	6,670	6,600	6,930	6,840	5,200	6,230	9,050	8,930	6,670	7,030	7,840	8,050
60 min.	5,130	6,580	6,900	6,850	5,200	6,190	8,870	8,880	4,800	7,060	7,770	8,000
% decrement	-23.0	-0.2	-0.5	+0.2	0.0	-0.7	-1.8	-0.4	-28.0	+0.5	-1.1	-0.6

\* Both plasmas stored at 10°C for 10 days.

5. Tuberculous plasma, after storage at 10°C for 7 days, retains its capacity for lysing white cells in the presence of "Old Tuberculin" (Table III).

*Discussion.* An earlier report<sup>6</sup> stressed the essential role of tuberculous plasma in the one-hour *in vitro* cytotoxicity of human white cells. The foregoing results now indicate that the active component of such tuberculous plasma is a non-dialyzable, heat-labile fraction which remains stable after storage for 7 days at 10°C. The inactivation of such plasma by heating to 56°C for 15 minutes is apparently not due to the destruction of complement since the addition of fresh guinea pig complement did not restore the leucocyte lysing activity of the heated plasma. The precipitation of this active factor by dialyzing tuberculous plasma against distilled water seems to place this lysing component in the globulin fraction of the plasma proteins. The heat lability of this plasma substance distinguishes it from the usual precipitating and agglutinat-

ing antibodies described in tuberculous plasma. It is of particular interest that Chase<sup>4</sup> was able to destroy the ability of tuberculous cells to transfer sensitivity passively if such cells were heated to 48° for 15 minutes. In fact, work now in progress suggests that such tuberculous cells may be the source of this lytic promoting factor in plasma. It is not known whether the cytolytic process herein described represents an *in vitro* counterpart of the tuberculin type tissue reaction.

*Summary.* There is present in the plasma of tuberculous humans a heat-labile, non-dialyzable component which is responsible for the *in vitro* lysis of washed leucocytes by tuberculo-protein. Further properties of this lytic producing substance are its stability at 10°C for at least 7 days and its precipitation with the globulin fraction of the plasma proteins.

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## 17167. The Precipitating Effect of Methanol on Viruses.\*

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Many technics have been applied to the problem of concentration of viruses. Those which utilize chemical and physical methods have been well reviewed by others.<sup>1-3</sup> Recently Cox *et al.*<sup>4</sup> reported on the application of the protein-precipitating property of methyl alcohol to the concentration of influenza B virus. By virtue of what seems to be a denaturing effect on somatic proteins and an innocuousness for virus protein, this technic appears to offer some promise of providing a method which might be useful with a wide range of viruses. It requires careful control over methanol concentration, temperature of reaction, and pH of the elution fluid.

The technic of virus precipitation with methanol is described in detail by Cox *et al.* and the procedure used in this study is essentially unchanged. The virus suspension is maintained at 0°C to 5°C in an ice water bath. Chilled methanol (C.P.) is slowly mixed into the virus suspension and shortly thereafter a precipitate appears. The precipitation reaction is permitted to proceed for 3 hours. The precipitate is then sedimented in a chilled angle centrifuge at 4600 rpm for 30 minutes. The supernatant fluid is tested for virus activity and, since it is usually relatively free of virus, it is then discarded. The precipitate is resuspended to any volume desired in 0.2 M citrate or phosphate buffer, and the virus is permitted to elute at room temperature for one hour. The suspension is then centrifuged at 2500 rpm for 15 minutes at room temperature. The resulting

supernatant fluid contains most of the virus.

Five viruses† were studied with this methanol precipitation technic, *viz.*: 10% saline emulsion of Eastern equine encephalomyelitis virus (EEE) in mouse brain; 10% saline emulsion of human poliomyelitis virus (MEF) in mouse spinal cord; and influenza B, Newcastle disease, and mumps viruses in allantoic fluid. The EEE and MEF viruses were tested in decimal dilutions (0.03 ml per mouse) for infectivity by intracerebral inoculation into 10 g Swiss mice. Fifty percent lethal end points were determined according to the method described by Reed and Muench.<sup>5</sup> The 3 remaining viruses were tested for activity by the Hirst technic of chicken rbc agglutination.<sup>6</sup> In studying these five viruses some uniformity of pattern seems to have been manifested, particularly concerning the optimum concentration of methanol, and the optimum pH level at which virus elution occurs. All experiments re-

TABLE I.  
Methanol Concentration and Elution pH for  
Optimal Yield of Virus.

Virus	Optimum	
	% methanol	Elution pH
EEE	25	7
MEF	25	8
Mumps	30	8
Influenza B	25	9
Newcastle disease	30	7

† Source of viruses:

MEF poliomyelitis—Dr. P. K. Olitsky, Rockefeller Institute, New York, N. Y.

Eastern equine encephalomyelitis—Local outbreak in equines during fall of 1947.

Mumps—Dr. J. F. Enders, Children's Hospital, Boston, Mass.

Influenza B—Dr. Joseph E. Smadel, Army Medical School, Washington, D.C.

Newcastle disease—Dr. L. T. Giltner, U.S.B.A.I., Pathological Division, Washington, D.C.

<sup>5</sup> Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27**, 493.

<sup>6</sup> Hirst, G. K., *Science*, 1941, **94**, 22.

\* Supported by a grant from the National Foundation for Infantile Paralysis, Inc.

<sup>1</sup> Anderson, T. F., *Cold Spring Harbor Symposium on Quantitative Biology*, 1946, **11**, 1.

<sup>2</sup> Pirie, N. W., *Annual Rev. of Biochem.*, 1946, **15**, 573.

<sup>3</sup> Gard, Sven, *Acta Med. Scand. Supp.*, 1943, **143**, 1.

<sup>4</sup> Cox, H. R., Van der Scheer, J., Aiston, S., and Boluel, E., *J. Immunol.*, 1947, **50**, 148.

## DETERMINATION OF OPTIMUM METHANOL CONCENTRATION FOR PRECIPITATION OF VIRUSES

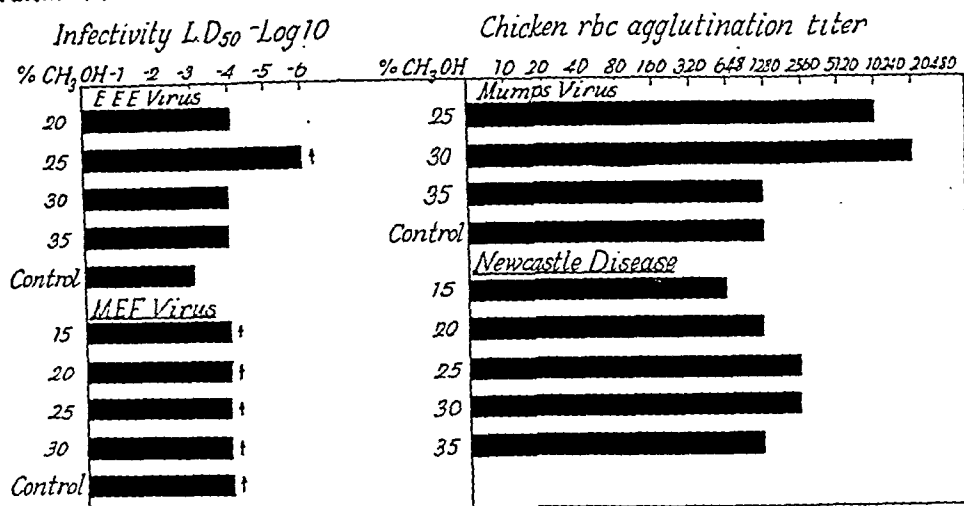


Fig. 1.

ported herein were repeated at least once, and the results were sufficiently in accord so as to warrant being published.

The optimum concentration of methanol was determined by adding 20, 25, 30, and 35% of methanol to aliquots of the virus suspension. By testing the resulting virus eluates in pH 7 buffer, the optimum methanol concentrations were arrived at and are listed in Table I: 25% methanol was optimal for EEE and MEF;† 30% for mumps virus and Newcastle disease virus. The concentration of methanol to be used for MEF virus was determined on the basis of complete extraction of the agent from the suspension by the precipitate and on this basis, 25% was found to be optimal. (Fig. 1).§ In accord with a previous report,<sup>1</sup> 25% methanol concentration was found to be optimal for influenza B virus.

Using the optimum concentration of methanol, as determined above for each virus, the pH at which each of the viruses would be eluted from the precipitate (or redissolved)

† In a recent report Gollan indicates that 33% methanol was suitable for precipitating rodent MM poliomyelitis virus, *Proc. Soc. Exp. Biol. and Med.*, May 1948, 67, 3.

§ The arrows in Fig. 1 and III indicate that an end point was not determined.

most effectively was determined (Table I). It can be noted that the eluates were more potent as the pH of the eluting fluid was raised (Fig. 2). Each of the 5 viruses appeared to be more soluble in buffers at pH 7 or above than with buffers under pH 7. It was thought that some viruses might be inactivated at the lower pH levels, and so explain the lower titers of the eluates. However, when a precipitate containing EEE virus was eluted at pH 5 and then reeluted at pH 7, the virus was absent in the first elution but was recovered in the second step.

When virus suspensions were precipitated with methanol, the final eluate was found to contain less nitrogen than the original suspension. When the virus suspension was concentrated by reducing the relative volume of the final eluate, there was no proportional increment in content of nitrogen, while the virus content per ml was considerably increased. In fact, while there was considerable loss of nitrogen as a result of methanol precipitation, this loss did not appear to be at the expense of the virus. Table II illustrates some of the results of such micro-kjeldahl determinations.

**Discussion.** It can be noted that these methanol treated viruses have yielded eluates which are inordinately more potent than the

## DETERMINATION OF OPTIMUM ELUTION pH OF METHANOL PRECIPITATED VIRUSES

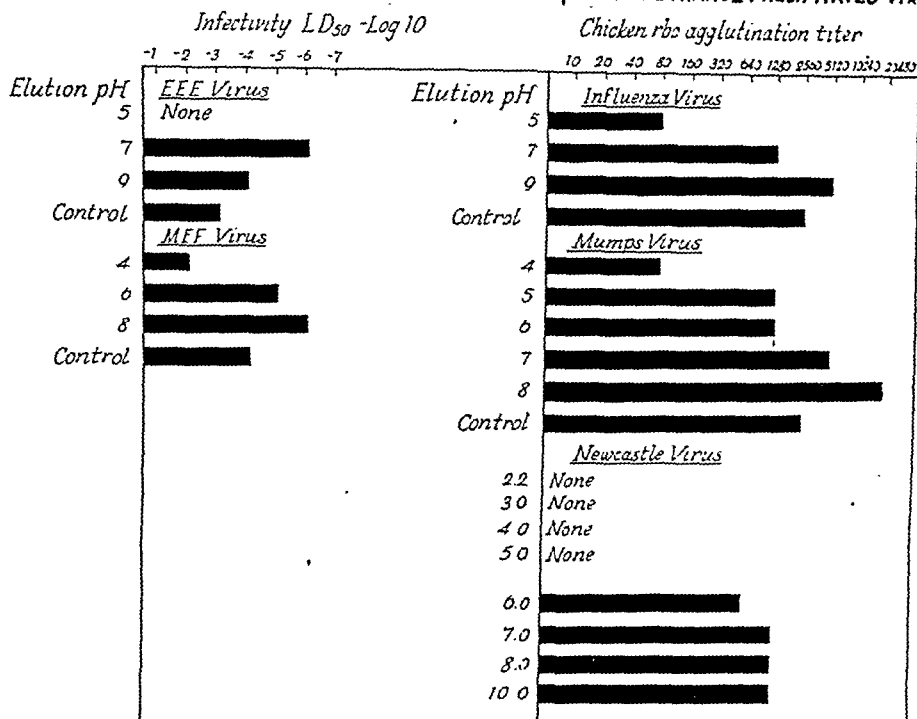


FIG. 2.

concentration factor would indicate. Our work has not yet offered any explanation for this phenomenon. The enhancement might be due to the disruption of infective aggregates or to the removal of some inhibiting substances as a result of this treatment with methanol. It may be that the viruses are manifesting an optimum pH activity point; however, Wenner<sup>7</sup> has reported that pH 4.6 is the optimum effective point for poliomyelitis. At this pH level, lower titratable end-points were demonstrated in the eluates from methanol precipitated virus suspensions than were attained at the higher pH levels (Fig. 2). Horsfall<sup>8</sup> refers to autointerference agents which might provide an explanation for the enhancement of potency of the viruses following treatment with methanol. In all in-

stances the nitrogen content of the virus suspension was reduced following methanol treatment, but not at the expense of the infective agent. This is in accord with the findings of Koprowski *et al.*<sup>9</sup> with methanol-precipitated viruses.

It may be that the viruses are less soluble at the lower pH levels because of the proximity to their isoelectric points; and in accordance with what has been demonstrated with enzyme proteins<sup>10</sup> and with animal serum proteins,<sup>11</sup> the solubility of the viruses increases as one moves away from this pH. As indicated above, while EEE virus was insoluble it was not destroyed after exposure

<sup>7</sup> Wenner, H. A., *Proc. Soc. Exp. Biol. and Med.*, 1945, 60, 104.

<sup>8</sup> Horsfall, Frank L., Jr., *Viral and Rickettsial Infections of Man*, J. B. Lippincott Co., Philadelphia, 1948.

<sup>9</sup> Koprowski, H., Black, J., and Cox, H. R., *Proc. 4th Internat. Congress for Microbiol.*, July 1947.

<sup>10</sup> Northrop, J. H., *Crystalline enzymes*, Columbia University Press, 1939.

<sup>11</sup> Cohn, E. J., and Edsall, G., *Proteins, Amino Acids and Peptides*, Reinhold Publishing Corp., New York, 1943.

TABLE II.  
Nitrogen Loss by Precipitation of Virus Suspension with Methanol.

Virus CAF*	Mg N crude	Fold. conc.	Mg N after precipitation	% N reduction
Newcastle	.64	0	0.20	68.8
"	.62	0	0.35	43.8
"	.62	10	0.42	99.2
Influenza B	.54	0	0.21	62.0
" A	.47	0	0.14	70.3
" A	.47	10	1.12	76.2
Mumps	.56	100	2.91	94.9
"	.56	100	1.47	97.3
"	.56	100	0.35	99.3

\* CAF—Chorio-allantoic fluid.

to pH 5 for one hour, and it was thereafter recovered with appropriate pH solutions. It has been reported that poliomyelitis virus can tolerate exposure to pH 1.6,<sup>12</sup> and on this basis it seems probable that the MEF virus was not destroyed when it failed to elute at the lower pH levels. Additional studies of the pH stability of MEF poliomyelitis virus indicate that while the virus was most stable at pH 6-7 it was able to tolerate pH 3 for 10 weeks.<sup>13</sup>

*Summary.* Five viruses (Eastern equine

<sup>12</sup> Loring H., and Schwerdt, C. E., *Proc. Soc. Exp. Biol. and Med.*, 1944, **57**, 173.

<sup>13</sup> Pollard, M., and Connolly, J., *Tex. Reports on Biol. and Med.*, Spring 1949.

encephalomyelitis, MEF human poliomyelitis, mumps, influenza B, and Newcastle disease) were utilized in studying the precipitating effect on them of methanol. Optimal methanol concentrations and optimal pH for elution were determined for each virus and are tabulated (Table I). All of the viruses which were studied demonstrated a relationship between the pH of the elution fluid and solubility: as the pH was raised there was a corresponding increase in solubility of the virus. The methanol precipitation technic results in considerable loss of nitrogen from the virus suspension, but this loss does not appear to be at the expense of the virus.

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# 17168. Comparison of Newcastle Virus in Hamsters Exposed by Intracerebral Injection and Intranasal Instillation.

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Hamster-adapted Newcastle virus, California strain No. 11,914, has been continued through the 300th passage by serial intracerebral inoculation. Virus neutralization tests were conducted, using brain suspensions of the 245th and 300th intracerebral hamster passages with specific Newcastle virus immune and normal chicken sera, and with hamsters as test animals. Specific immune sera completely neutralized the hamster brain virus, whereas normal chicken serum had no effect. The virus of this 300th passage titred  $10^{-4.77}$  intracerebrally in 4-week-old hamsters,

in contrast to the virus of the 200th passage<sup>1</sup> which titred  $10^{-4.25}$  in hamsters of the same age injected by the same route.

Newcastle virus infection in hamsters inoculated intranasally with 10% brain suspension of the 16th intracerebral hamster passage has been previously reported.<sup>2</sup> It was also reported that intranasal instillations

<sup>1</sup> Reagan, R. L., Lillie, M. G., Hauser, J. E., and Brueckner, A. L., *Proc. Soc. Exp. Biol. and Med.*, 1948, **68**, 293.

<sup>2</sup> Reagan, R. L., Lillie, M. G., Poelma, L. J., and Brueckner, A. L., *Am. J. Vet. Res.*, **8**, 427.

TABLE I.  
Intranasal Passage of Hamster-Adapted Newcastle Virus.

Passage No.	No. inoculated	No. showing symptoms	No. days after inoculation symptoms occurred
1	6	5	3, 3, 4, 4, 5
2	5	5	2, 2, 3, 3, 4
3	8	6	4, 4, 4, 4, 5, 5
3a*	5	2	3, 3
4	6	6	4, 4, 6, 6, 6, 6
4a	6	2	4, 5
5	4	1	3
6	4	1	4
7	5	3	4, 4, 5
7a	6	2	4, 4
8	4	2	3, 4
9	5	0	
9a	8	8	3, 4, 4, 4, 4, 4, 4, 4

\* "a"—Duplicate passages made from suspensions which were frozen and thawed.

TABLE II.  
Titration in 4-Week-Old Hamsters of Hamster-Adapted Newcastle Virus.

Virus	Dilutions					Titer <sup>3</sup>
	10-1	10-2	10-3	10-4	10-5	
Hamster brain 300th passage	—	4/4	4/4	3/4	2/4	10-4.77
Hamster cord 300th passage	4/4	4/4	0/4	0/4		10-2.5
Hamster brain from 4th intranasal passage	4/4	4/4	4/4	3/4	2/4	10-4.77
Hamster brain from 7th intranasal passage	6/6	4/4	4/4	1/4		10-3.66

Numerator of fraction denotes number of deaths.

Denominator of fraction denotes number of hamsters inoculated.

of lung or brain suspension from the affected animals failed to produce symptoms of central nervous system involvement in hamsters of the primary passage. Repeated trials with intranasal instillation of 10-20% infected brain suspension after the 16th intracerebral passage and up to the 245th intracerebral passage failed to produce typical symptoms of Newcastle virus infection, except in the first exposure. When a 20% brain suspension of the 245th intracerebral subculture was passed intranasally in young hamsters, symptoms involving the central nervous system, similar to those observed after intracerebral injection of the hamster-adapted Newcastle virus, were observed. These intranasal instillations were continued through 9 uninterrupted passages. Virus neutralization tests were conducted, using brain suspension of this 9th passage with specific Newcastle virus

immune and normal chicken sera, with hamsters as test animals. Specific immune serum completely neutralized the hamster brain virus while normal chicken serum had no effect. Symptoms of central nervous system involvement appeared between the 2nd and 6th days following intranasal exposure. Table I gives the results of these 9 passages. Table II gives results of titrations in 4-week-old hamsters of the hamster-adapted Newcastle virus from various sources.

*Discussion.* Similar symptoms were shown by hamsters infected, by the intracerebral route and by intranasal instillation, despite the fact that the incubation period was much shorter by the first method. Virus of the higher numbered intracerebral passages, and

<sup>3</sup> Reed, L. J., and Muench, H., *Am. J. Hyg.*, 27, 493.

including the 300th, produced marked nervous reactions within 24 hours after intracerebral injection. This was true when dilution was carried as high as 1 to 1000. Hamsters exposed by intranasal instillation of 20% brain suspension in amounts as small as 0.06 cc became infected. In exposure by intranasal instillation the incubation period was regularly increased and ranged from 2 to 6 days. Brain suspension from hamsters infected intranasally in the 4th passage titred  $10^{-4.77}$  by intracerebral injection, whereas the same form of material from the 7th passage titred  $10^{-3.66}$  by the same route. These titrations were made with 0.03 cc of the various brain suspension dilutions. Spinal cord of hamsters of the 300th intracerebral passage, injected in 10% suspension intracerebrally, produced typical nervous symptoms. This suspension of virus titred  $10^{-2.5}$ , as shown in Table II.

*Summary.* Infected hamster brains from

the 300th intracerebral passage and from the 4th intranasal passage titred  $10^{-4.77}$ , whereas infected brains from the 7th intranasal passage titred  $10^{-3.66}$ . Positive Newcastle chicken serum neutralized the virus from the 245th and 300th hamster passage, whereas normal chicken serum had no effect.

Nasal instillation trials were conducted from the 16th and 300th hamster passage but were not successful (except for the 1st passage) until after the 245th passage.

The incubation period of hamsters infected by intranasal instillation is longer than by intracerebral injection. Hamster-adapted Newcastle virus was carried through 9 passages intranasally, and brain material from this 9th nasal passage was neutralized by positive Newcastle chicken serum. Hamsters infected intranasally and intracerebrally showed symptoms of irritability followed by involuntary motor reactions and paralysis.

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### 17169. The Stability and the Stabilization of Testicular Hyaluronidase.

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Over a period of years, numerous preparations of hyaluronidase have been stored in these Laboratories as bulk powder at room temperature without any appreciable loss in activity. It has been observed, however, that decreases in potency occur when the enzyme is stored under other conditions. We have therefore conducted an investigation of the factors which influence the stability of testicular hyaluronidase. This involved the development of an accelerated aging test and the use of stabilizing agents. The stability of hyaluronidase in bulk, in aqueous solution and in sterile vials has been studied at 10°C, at room temperature and under conditions of accelerated aging. The influence of various bacteriostatic agents on the lability of the enzyme is reported.

*Methods.* The turbidimetric method of

assay employed in this investigation has recently been described in detail.<sup>1</sup> This procedure permits the use of hyaluronates of varying degrees of purity as substrates and gives consistent results because of the stabilization of the protein indicator and the prompt termination of the reaction by heat. The activity of the enzyme is expressed in turbidity reducing units (T.R.U.) which are defined as that amount of enzyme which will hydrolyze hyaluronic acid until it gives a turbidity corresponding to half the amount of substrate employed. Using this method of assay the units have numerical values lower than those obtained by other procedures: *e.g.* 100 TRU

<sup>1</sup> Tolksdorf, S., McCready, M. H., McCullagh, D. Roy, and Schwenk, E., *J. Lab. Clin. Med.*, 1949, 34, 74.



TABLE I.  
Loss in Activity of Bulk Hyaluronidase.

Potency of sample TRU/mg	10°C		25-30°C		60°C	
	Time in mo.	% loss	Time in mo.	% loss	Time in mo.	% loss
25	12	0	12	0	1	40
150	12	0	12	0	1	20
500	2	0	3	58	1	62

by this method might be as high as 200 TRU by other methods.

The bulk hyaluronidase was prepared from bull testes by ammonium sulfate and lead acetate fractionation<sup>2,3</sup> and dried from the frozen state. Concentrated solutions of hyaluronidase in pyrogen-free distilled water were sterilized by Seitz filtration and tested for stability at various dilutions. Dilute, sterile filtrates of hyaluronidase were dried in vials from the frozen state. Early experiments indicated that hyaluronidase was less stable in vials than in bulk. Human plasma was added to the hyaluronidase and found to be a suitable stabilizer. Its use however was discontinued due to possible contamination with the virus of infectious hepatitis. It has been noted that certain labile substances such as protein hormones could be stabilized by the use of gelatine.<sup>4</sup> Gelatine which had been purified for intravenous use was therefore added to the vials before drying. This material after drying could be reconstituted only with difficulty. A polypeptide derived from gelatine\* was then employed.

The shelf life of hyaluronidase both with and without stabilizer was determined upon storage at room temperature and at 10°C. Since some of the preparations proved to be very stable, an accelerated aging test was devised in order to expedite the study of the stabilization of hyaluronidase. The increased rate of inactivation was studied at various

elevated temperatures up to 60°C. Although hyaluronidase in aqueous solution is destroyed almost immediately at 60°C,<sup>1</sup> hyaluronidase in dry form is inactivated at this temperature at a rapid but measurable rate. The stability of bulk powder as well as that of the enzyme in vials was therefore determined by incubation at 60°C for various periods of time.

Hyaluronidase in solution and dried in vials was also tested for stability in the presence of such bacteriostatic agents as zephiran (alkyl dimethyl benzyl ammonium chlorides), phenyl mercuric nitrate and tegosept (methyl and propyl esters of para hydroxy benzoic acid).

*Results. I. Stability of Bulk Hyaluronidase.* The stability of bulk hyaluronidase is illustrated in Table I. Material assaying 25 TRU and 150 TRU per mg respectively was stable for more than one year when stored at 10°C and at room temperature. Material of higher potency (500 TRU per mg) was stable for several months at 10°C. Stored at room temperature, the 500 TRU material lost 58% of its activity in 3 months. It should be mentioned however, that this study was carried out between June and September, 1948, when the average room temperature was close to 30°C. At 60°C the bulk material, regardless of potency, lost considerable activity over a period of one month.

*II. Stability of Aqueous Solutions of Hyaluronidase.* Aqueous solutions of hyaluronidase in 2% concentration possessed remarkable stability at 10°C. As may be seen from Table II, sterile filtrates of an enzyme preparation assaying 150 TRU per mg were stable for more than 3 months when kept in the refrigerator.

The stability of dilute enzyme solutions was studied at 10°C with 2 preparations assaying 150 and 500 TRU per mg respectively. The

<sup>2</sup> Madinaveitia, J., *Biochem. J.*, 1941, **35**, 447.

<sup>3</sup> Kuhn, L., *Biochem. Z.*, 1943, **315**, 83.

<sup>4</sup> Bockmühl, M., Lindner, F., and Schaumann, O., U. S. Patent No. 2,050,558, 1936.

\* We are grateful to Dr. D. Tourtellotte of the Knox Gelatine Protein Products, Inc., Camden, N. J., who supplied the first experimental batches of intravenous gelatine and informed us of the availability of, and supplied us with, the polypeptide.

TABLE II  
Stability of Concentrated Aqueous Solutions of Hyaluronidase at 10°C.

Experimental*	Original assay, TRU/ml	Time at 10°C, mo.	Final assay, TRU/ml
STE8-gen-20	2580	3	2730
" 22	2640	3	2760
STE9-gen-1B	2590	3	2700
" 6	2670	2.25	2900
" 9	3220	2.5	3260

\* Enzyme employed assayed 150 TRU/mg. Concentration of sterile filtrates approximately 2%.

TABLE III  
Loss of Activity of a Dilute Aqueous Hyaluronidase Solution with and without Stabilizer.

Enzyme STII-24*	Without stabilizer		With polypeptide	
	Time	Loss, %	Time	Loss, %
10°	7 wks	50	7 wks	0
Room temp.	7 days	50	18 days	50

\* Starting material 500 TRU/mg in 0.028% solution (140 TRU/ml).

500 TRU material at a concentration of 0.28 mg per ml lost 50% of its activity in 7 weeks whereas at the same temperature, in the presence of 2 mg of polypeptide per ml, there was no loss in activity over the same period of time (Table III). At room temperature, the half-life of the enzyme was 7 days without stabilizer as compared to a half-life of 18 days in the presence of polypeptide.

*III. Stability of Hyaluronidase in Vials.* The lability of hyaluronidase dried from the frozen state in vials in the absence of stabilizer was studied using material assaying 150 TRU per mg and 500 TRU per mg. The vials contained either 5 to 10 TRU, 100 TRU or approximately 150 TRU of the enzyme and were stored at 10°C, at room temperature and at 60°C. Regardless of the amount of hyaluronidase in the vials, the enzyme was stable at 10°C for more than 7 months. At room temperature, losses were frequently observed after 4 months while at 60°C, losses were observed within hours.

The addition of gelatine resulted in a very marked increase in the stability of the enzyme. For vials containing 5 TRU of hyaluronidase, the half-life was 2.5 hours at 60°C without gelatine whereas there was only a very slight loss in the potency of the same preparation in 12 days in the presence of 2 mg of this protein. Unfortunately the contents of the vials were not readily soluble in distilled water.

It was found that a polypeptide produced

by the partial hydrolysis of gelatine\* was quite soluble after being dried from the frozen state. The stabilizing effects of this polypeptide were the same over a range of 1 to 7.5 mg per vial. Therefore, it was decided to use 2 mg per vial regardless of the quantity and purity of the enzyme. In the presence of this stabilizer the hyaluronidase showed no loss of activity at 10°C and at room temperature for more than 6 months. Some studies made at 60°C are demonstrated in Table IV and are typical of our findings. The results given in the table are expressed in terms of half-life of the enzyme. It will be seen that the half-life of 500 TRU material under these conditions was increased 16 fold. The 150 TRU material had an average increase in half-life from 6.9 to 156 days, indicating a 23 fold increase in stability due to the polypeptide. The vials of batch number 8-gen-17 E contained only small quantities of the enzyme but were stabilized to the same extent as were the other batches. More highly purified hyaluronidase (FVIV-38 C) lost all activity during drying in the absence of stabilizer when the charge was 10 micrograms representing 5 TRU. No activity was lost in the presence of polypeptide. Since the enzyme without polypeptide was stable at room temperature in vials for a period of 4 months, it would seem possible that in the presence of polypeptide the stability at room temperature might be increased to a period of years.

TABLE IV.  
Half-Life of Hyaluronidase in Vials at 60°C.

Batch No.	Potency	Vials filled to contain	Half-life	
			Without stabilizer, days	With stabilizer, days
FV IV-38 A	500 TRU/mg	150 TRU/vial	3.5	56
8-gen-17 C	150 " "	165 " "	4.6	163
8-gen-17 A	150 " "	110 " "	14	114
8-gen-17 E	150 " "	9 " "	2	190
FV IV-38 C	500 " "	5 " "	*	85

\* All activity lost during drying.

TABLE V.  
Stability of Hyaluronidase in Vials During Freeze-Drying.

Batch No	Potency	Content of vials before drying, TRU	Content of vials after drying	
			Without stabilizer, TRU	With stabilizer, TRU
FV IV-38 A	500 TRU/mg	150	120	160
8-gen-17 C	150 " "	165	154	163
8-gen-17 A	150 " "	110	98	114
8-gen-17 E	150 " "	9	4.7	11
FV IV-38 C	500 " "	5	no activity	5.7

Observations were made to determine whether or not a decrease in potency occurred while drying the enzyme in vials. The influence of polypeptide on possible losses during drying was also investigated. Five such experiments are summarized in Table V. In each experiment the same amount of enzyme was placed in the vials with stabilizer as in the control vials. It will be noted that in the absence of stabilizer, losses occurred which increased as the amount of enzyme decreased. In the presence of 2 mg of polypeptide, these losses were not observed. During the investigation of solutions of hyaluronidase, it had been noted that the polypeptide did not influence the initial assay values. Therefore it is believed that these experiments indicate that the polypeptide exerts a protective influence over the enzyme during drying.

*IV. The influence of Bacteriostatic Agents on the Stability of Hyaluronidase.* Tegosept, zephiran and phenylmercuric nitrate were employed in maintaining bacteriostasis in preparing various batches of hyaluronidase. These

substances were separated from the enzyme by dialysis prior to drying the final product from the frozen state. Satisfactory preparations were obtained using zephiran and phenylmercuric nitrate. Some preparations in which tegosept was employed became inactive.

*Summary.* 1. Testicular hyaluronidase in bulk assaying 150 TRU per mg is stable at room temperature for more than one year.

2. Hyaluronidase in concentrated aqueous solution is stable for several months at 10°C. Dilute solutions of the enzyme are less stable.

3. Hyaluronidase dried from the frozen state in vials can be stabilized by the addition of polypeptide. Its half-life at 60°C is thereby extended 10 to 20 times.

4. Certain bacteriostatic agents can be used throughout the production of hyaluronidase without decreasing the activity of the enzyme.

The authors are grateful to Dr. Erwin Schwenk for his encouragement throughout this investigation.

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## 17170. Sympatholytic Effects of Diethylaminoethanol in Man.\*

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(Introduced by Robert W. Wilkins.)*From the Robert Dawson Evans Memorial, Massachusetts Memorial Hospitals, and the Department of Medicine, Boston University School of Medicine, Boston, Mass.*

A recent report by Rosenberg and his co-workers<sup>1</sup> indicates that diethylaminoethanol, an *in vivo* decomposition product of procaine, may revert ventricular tachycardia to normal rhythm, suppress ventricular premature contractions, and induce transient hypotension. Since the mechanism of these effects was not determined the present investigation was instituted to inquire into the hemodynamic properties of the drug in man.

**Methods and results.** Both hypertensive and normotensive subjects were given diethylaminoethanol in doses of 2 to 5 g either by slow intravenous drip in 0.5% solution at a rate of 0.02 g per minute or by rapid intravenous injection of a 10% solution at a rate of 1.0 g per minute. Sympathetic vasopressor and digital vasoconstrictor reflexes were determined before and at various intervals after the drug according to methods previously described.<sup>2</sup> Doses of 5 g of diethylaminoethanol injected intravenously at a rate of 1 g per minute were followed by a reduction of basal arterial pressure and marked postural hypotension. Heart rate either did not change significantly or increased moderately. Accompanying the hypotensive response there was an inhibition of sympathetic vasopressor

responses (Valsalva and tilt-back overshoots), as well as a reduction in reflex vasoconstrictor responses to "noxious stimuli" in the digits. In addition, a sudden increase in digital volume and pulse volume was observed. These hypotensive and sympatholytic effects were transient, the arterial pressure and sympathetic reflexes returning to normal within  $\frac{1}{2}$  to 1 hour after the drug was administered. Slow rates of administration of the drug and low dosage were not followed by such cardiovascular or sympathetic reflex changes. No serious toxic effects were noted.

**Discussion.** The observed sympatholytic properties of diethylaminoethanol in man probably are the basis for its hypotensive action as well as its effect on ventricular tachycardias. In the latter respect the drug is similar to certain other sympatholytic agents such as dibenamine,<sup>3</sup> ergotamine,<sup>4</sup> and dihydroergokryptine.<sup>5</sup> This correlation between adrenergic inhibition and the prevention or cessation of attacks of ventricular tachycardia seems to be limited to drugs which suppress the sympathetic system only: since in dogs under cyclopropane anesthesia the drug tetraethylammonium, which inhibits transmission through all autonomic ganglia, intensifies rather than prevents epinephrine induced ventricular tachycardia.<sup>6</sup>

Rosenberg and his co-workers demonstrated that high blood levels of diethylaminoethanol are not maintained due to rapid diffusion of the drug out of the blood stream.<sup>1</sup> The results of the present study suggest that the sympha-

\* This investigation was supported in part by the Squibb Institute for Medical Research, New Brunswick, N. J. The diethylaminoethanol used in this investigation was generously supplied by Dr. James A. Shannon of the Squibb Institute.

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<sup>‡</sup> On leave of absence from the Mary Hitchcock Memorial Hospital and Dartmouth Medical School, Hanover, N. H.

<sup>1</sup> Rosenberg, B., Kayden, H. J., Lief, P. A., Marks, L. C., Steele, J. M., and Brodie, B. B., *J. Pharm. and Exp. Ther.*, 1949, 95, 18.

<sup>2</sup> Freis, E. D., Stanton, J. R., Culbertson, J. W., Halperin, M. D., Litter, J., Burnett, C. H., and Wilkins, R. W., *J. Clin. Invest.*, 1949, 28, 353.

<sup>3</sup> Nickerson, M., and Goodman, L. S., *Fed. Proc.*, 1948, 7, 397.

<sup>4</sup> Allen, C. R., Stutzman, J. W., and Meek, W. J., *Anesthesiology*, 1940, 1, 158.

<sup>5</sup> Stanton, J. R., Ware, P., and Stutzman, J., unpublished data.

<sup>6</sup> Stutzman, J. W., Pettinga, F. L., Fruggiero, E. J., and Maison, G. L., *Proc. Soc. Exp. Biol. and Med.*, 1945, 68, 686.

TABLE IV.  
Half-Life of Hyaluronidase in Vials at 60°C.

Batch No.	Potency	Vials filled to contain	Half-life	
			Without stabilizer, days	With stabilizer, days
FV IV-38 A	500 TRU/mg	150 TRU/vial	3.5	56
8-gen-17 C	150 " "	165 " "	4.6	163
8-gen-17 A	150 " "	110 " "	14	114
8-gen-17 E	150 " "	9 " "	2	190
FV IV-38 C	500 " "	5 " "	*	85

\* All activity lost during drying.

TABLE V.  
Stability of Hyaluronidase in Vials During Freeze-Drying.

Batch No	Potency	Content of vials before drying, TRU	Content of vials after drying	
			Without stabilizer, TRU	With stabilizer, TRU
FV IV-38 A	500 TRU/mg	150	120	160
8-gen-17 C	150 " "	165	154	163
8-gen-17 A	150 " "	110	98	114
8-gen-17 E	150 " "	9	4.7	11
FV IV-38 C	500 " "	5	no activity	5.7

Observations were made to determine whether or not a decrease in potency occurred while drying the enzyme in vials. The influence of polypeptide on possible losses during drying was also investigated. Five such experiments are summarized in Table V. In each experiment the same amount of enzyme was placed in the vials with stabilizer as in the control vials. It will be noted that in the absence of stabilizer, losses occurred which increased as the amount of enzyme decreased. In the presence of 2 mg of polypeptide, these losses were not observed. During the investigation of solutions of hyaluronidase, it had been noted that the polypeptide did not influence the initial assay values. Therefore it is believed that these experiments indicate that the polypeptide exerts a protective influence over the enzyme during drying.

IV. *The influence of Bacteriostatic Agents on the Stability of Hyaluronidase.* Tegosept, zephiran and phenylmercuric nitrate were employed in maintaining bacteriostasis in preparing various batches of hyaluronidase. These

substances were separated from the enzyme by dialysis prior to drying the final product from the frozen state. Satisfactory preparations were obtained using zephiran and phenylmercuric nitrate. Some preparations in which tegosept was employed became inactive.

*Summary.* 1. Testicular hyaluronidase in bulk assaying 150 TRU per mg is stable at room temperature for more than one year.

2. Hyaluronidase in concentrated aqueous solution is stable for several months at 10°C. Dilute solutions of the enzyme are less stable.

3. Hyaluronidase dried from the frozen state in vials can be stabilized by the addition of polypeptide. Its half-life at 60°C is thereby extended 10 to 20 times.

4. Certain bacteriostatic agents can be used throughout the production of hyaluronidase without decreasing the activity of the enzyme.

The authors are grateful to Dr. Erwin Schwenk for his encouragement throughout this investigation.

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TABLE I.  
Relative *in vitro* Spasmolytic Properties of WIN 1539 and Demerol.

Compound	Barium chloride induced spasms		Acetylcholine induced spasms		Histamine induced spasms	
	Effective dilution*	Relative activity % papaverine	Effective dilution*	Relative activity % atropine SO <sub>4</sub>	Effective dilution*	Relative activity % papaverine
WIN 1539	1:1,000,000	639	1:3,200,000	2.5	1:1,000,000	475
Demerol	1:300,000	200	1:98,000	0.08	1:205,000	98

\* Values are averages from intestinal strips of 6 animals.

were injected into the exposed femoral vein. A total of 6 dogs was used in these experiments. The most marked effect on the intestine following the administration of WIN 1539 was a decrease in tonus. There was no significant change in amplitude or frequency of the contractions. Doses of WIN 1539 as low as 0.01 mg/kg produced a definite decrease in tonus. These small doses had no effect on blood pressure, heart rate or respiration. Larger doses (1-2 mg/kg) did not cause stimulation of the intestine but only a relaxation of longer duration. The results obtained in a representative experiment are shown in Fig. 1.

C. Roentgenographic study of the rate of movement of a barium meal through the gastrointestinal tract. The method used was a modification of the one described by Gershon-Cohen and Shay.<sup>7</sup> Adult male albino rats were starved for approximately 24 hours. They were then given orally a 50% suspension of barium sulfate in 0.5% gum tragacanth, by intubation in a dose of 0.5 cc/100 g of body weight. The analgesic compounds were injected subcutaneously as aqueous solutions immediately preceding the barium meal. The doses used are indicated in Table II. These doses had been found to produce approximately the same degree of analgesia in rats. Roentgenograms were made at 1, 2, 3 and 4 hours following the administration of the barium meal. The results are summarized in Table II. The time for complete evacuation was not determined, but the data obtained indicate that there is a delay in the

emptying time of the stomach due to the action of each of the analgesic drugs. Morphine caused the greatest and Demerol the least delay. The delay resulting from WIN 1539 was almost as great as from morphine. Morphine appears to act somewhat longer than WIN 1539 on the gastrointestinal tract which is also true of its analgesic effect. Karr<sup>8</sup> reported that morphine slowed the passage of a carbon suspension in the intestine of rats and that Demerol was much less active in this respect. This is undoubtedly related to

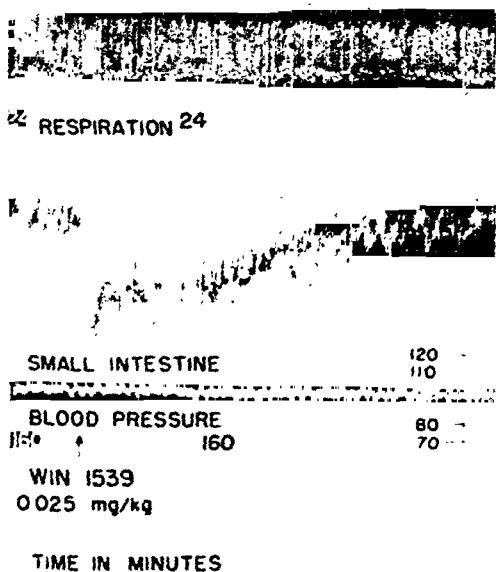


Fig. 1.  
Action of WIN 1539 in an anesthetized dog. Upper figures are respiratory rates. Lower figures are heart rates. Scale at right represents mm Hg pressure.

<sup>7</sup> Gershon-Cohen, J., and Shay, H., *The Rat in Laboratory Investigation*, 1942, p. 371. J. B. Lippincott Company, Philadelphia.

<sup>8</sup> Karr, N. W., *Fed. Proc.*, 1947, 6, 343.

tholytic and hypotensive effects are dependent upon a high blood level of the drug since slow rates of infusion at low dosage did not result in sympathetic inhibition. Even with large doses rapidly injected adrenergic inhibition was transient and disappeared at a rate similar to the observed disappearance of high concentrations of diethylaminoethanol from the blood. It was not possible to determine from the present experiments whether the site of sympathetic inhibition was central or peripheral.

*Summary and conclusions.* Doses of diethylaminoethanol sufficient to produce high blood

concentrations resulted in a temporary inhibition of sympathetic vasoconstrictor reflexes in man. It is suggested that the effects of the drug on arterial pressure and ventricular tachycardias are secondary to its sympatholytic action.

Since preparation of this paper, Clark and Helpern (*Fed. Proc.*, 1949, 8, 282) have reported on the ganglionic blocking action of diethylaminoethanol in dogs and cats. Their observations on the sympatholytic effects of the drug in animals are in essential agreement with the results of the present investigation in man.

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### 17171. Action of 1-Methyl-4-(3-Hydroxyphenyl)-4-Piperidyl Ethyl Ketone on the Gastrointestinal Tract.\*

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One of the most active analgesic compounds reported by Kleiderer, Rice *et al.*<sup>1</sup> was 1-methyl-4-(3-hydroxyphenyl)-4-piperidyl ethyl ketone which will be referred to in this report as WIN 1539. This compound has the same basic phenyl-piperidine nucleus as Demerol (brand of isonipecaine), but contains a meta-hydroxy group on the phenyl ring and has a propionyl group in place of the carbethoxy substituent of Demerol. The analgesic activity of WIN 1539 is 5-10 times greater than that of Demerol.<sup>1-4</sup> Because of its high analgesic activity and its close chemical re-

lationship to Demerol, the actions of WIN 1539 on the gastrointestinal tract have been investigated.

*Results. A. Effect on excised intestinal segments.* WIN 1539 and Demerol were compared for their activity against barium chloride and acetylcholine induced spasms of the rabbit ileum and against histamine induced spasms of the guinea pig ileum by the procedure described by Miller, Becker and Tainter.<sup>5</sup> The results are given in Table I. These data indicate that the spasmolytic activity of WIN 1539 is considerably greater than Demerol against all three spasmogenic agents.

*B. Effect on intestine in situ.* Kymographic recordings were made of a segment of the intact ileum of anesthetized dogs by a modification of the Barbour method as described by Jackson.<sup>6</sup> Aqueous solutions of the drugs

\* This work is part of an investigation carried out under the supervision of Dr. M. H. Seevers and submitted in a dissertation to the Graduate School of the University of Michigan in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

<sup>1</sup> Kleiderer, E. C., Rice, J. B., Conquest, V., and Williams, J. H., Report No. 981, Office of the Publication Board, Department of Commerce, Washington, D.C.

<sup>2</sup> Scott, C. C., Kohlstaedt, K. G., and Chen, K. K., *Anesth. and Analges.*, 1947, 26, 12.

<sup>3</sup> Lewis, J. R., Dissertation, University of Michigan, 1949.

<sup>4</sup> Cohen, R. L., Epstein, H. J., and Dramentz, C. S., *J. Pharmacol.*, 1948, 94, 328.

<sup>5</sup> Miller, L. C., Becker, T. S., and Tainter, M. L., *J. Pharmacol.*, 1948, 92, 260.

<sup>6</sup> Jackson, D. E., *Experimental Pharmacology and Materia Medica*, 1939, C. V. Mosby Co., St. Louis, Mo.

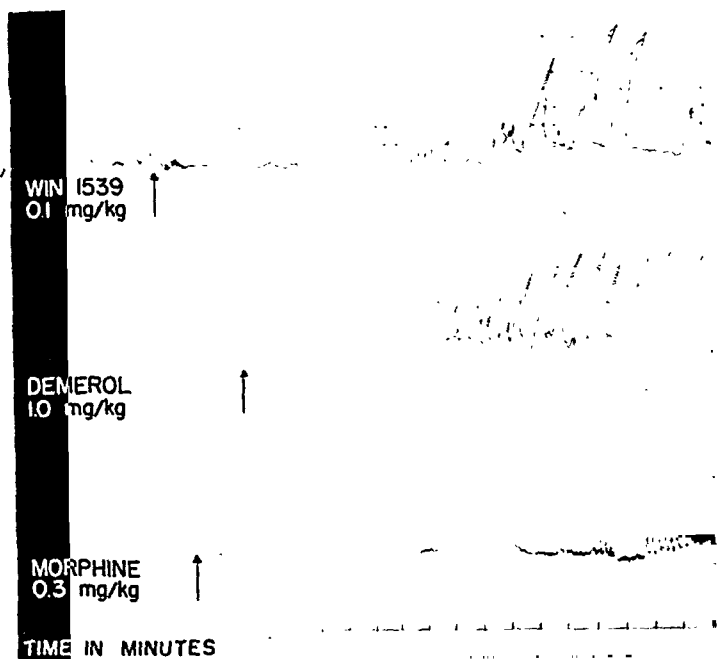


FIG. 2.  
Kymographic recordings of intact stomach of anesthetized rat.

but morphine retarded emptying more than either of these drugs. Demerol caused a stimulation of the intact rat's stomach whereas WIN 1539 and morphine decreased the motility of this organ.

The author wishes to express his sincere appreciation to Dr. J. O. Hoppe, Mr. J. D. Frick, Misses B. L. Dertinger and Estelle Ananenko for their assistance in this work.

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## 17172. Some Relationships of Folic Acid to Structurally Similar Metabolites.\*

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Jacobson and Good<sup>1</sup> observed that if folic acid (pteroylglutamic acid) is incubated with

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<sup>1</sup> Jacobson, W., and Good, P. M., *International Physiol. Congress, Abstracts*, 1947, July.

milk xanthopterin oxidase a material producing a greater response in cases of pernicious anemia than folic acid itself was obtained. At nearly the same time Kalckar and Klenow<sup>2</sup> demonstrated that xanthopterin is converted to leucopterin by milk xanthopterin oxidase and that this reaction is strongly inhibited by

<sup>2</sup> Kalckar, H. M., and Klenow, H., *J. Biol. Chem.*, 1948, **172**, 349.



TABLE II.  
Rate of Movement of a Barium Meal Through the Gastrointestinal Tract of Albino Rats.

Drug	No. of animals	Approximate % barium meal remaining in stomach Hr after administration				Location of remainder of barium meal 4 hr after administration
		1	2	3	4	
Untreated controls:	7					
Mean		15	9	7	4	
Range		2-50*	2-25	0-25	0-15	Cecum and feces.
Morphine (10 mg/kg):	5					
Mean		100	96	86	49	
Range		—	90-100	75-95	25-95	Jejunum and ileum.
Demerol (30 mg/kg):	9					
Mean		58	46	34	26	
Range		20-100	10-95	2-95	2-90	Lower ileum and cecum.
WIN 1539 (3 mg/kg):	10					
Mean		89	70	46	44	
Range		80-100	20-95	10-80	5-80	Jejunum, ileum and some in cecum of 4 animals.

\* 2% indicates a visible trace—other values are estimates of percentage of total volume as determined from area and density of barium meal.

the emptying time of the stomach as shown by our results.

D. *Effect on stomach in situ.* Since the roentgenographic study showed that the analgesic drugs retarded the emptying of the stomach, the effect of these drugs on the motility and tone of the intact stomach was investigated. Jackson's method<sup>6</sup> of recording contractions of the dog's stomach was modified for use with rats. The rats were anesthetized with sodium pentobarbital and the apparatus attached so that recordings of the pyloric portion of the stomach were obtained. The drugs were injected into an exposed saphenous vein.

A total of 12 animals were used in these experiments. Fig. 2 is a record obtained in a representative experiment. It was observed that WIN 1539 in a dose of 0.1 mg/kg caused a marked decrease in the motility of the stomach. In contrast, 1.0 mg/kg of Demerol produced an increase in tone and in some experiments also an increase in frequency of contraction. In two experiments morphine caused a response similar to that obtained with WIN 1539 in that the peristaltic waves were abolished.

The marked decrease in the motility of the stomach caused by WIN 1539 or morphine could account for the delay in the emptying

time which is an important factor in the constipating action of morphine. Gruber, Hart and Gruber<sup>9</sup> reported that Demerol produced a stimulation of the stomach and pylorus in anesthetized dogs. We obtained similar results in rats. In clinical use Demerol does not have a constipating effect. Even though morphine has an inhibitory action on the stomach of some species of animals (Krueger, *et al.*<sup>10</sup>), Veach<sup>11</sup> found that it is predominantly motor to the human stomach. Because of species differences in the digestive processes it cannot be stated that the actions obtained with WIN 1539 in these experiments would be the same for men.

*Summary.* WIN 1539, a new potent synthetic analgesic related chemically to Demerol, was found to have a stronger spasmolytic action than Demerol on the stimulated isolated intestinal strip. A decrease in tone of the intact small intestine of the dog was observed. WIN 1539 caused a greater delay than Demerol in the emptying time of the rat's stomach

<sup>9</sup> Gruber, C. M., Hart, E. R., and Gruber, C. M., Jr., *J. Pharmacol.*, 1941, **73**, 319.

<sup>10</sup> Krueger, H., Eddy, N. B., and Sumwalt, M., *The Pharmacology of the Opium Alkaloids*, U. S. Public Health Reports, Supplement No. 165, Part 1.

<sup>11</sup> Veach, H. O., *J. Pharmacol.*, 1937, **61**, 230.

thine and subsequent oxidation of the xanthine) is decreased about 50%. In the experiment shown aldehyde-free folic acid was employed although almost exactly similar results were obtained when commercial folic acid was employed. Because of the structural similarities between guanine and folic acid it appeared possible that the pterin nucleus could be metabolized similarly to guanine, *i.e.*, a hydrolysis and subsequent oxidation. However, such an effect as this involving oxidation would be obscured by the depression of endogenous respiration by folic acid. It is interesting to note that in the early portion of the curve for flasks containing folic acid there is a brief stimulation of oxygen uptake.

The similarity of the pterin nucleus to the flavin prosthetic groups and purines may possibly explain the depression of endogenous respiration and oxidation of the xanthine arising from guanine. It is also possible that

guanase itself is inhibited competitively by folic acid.

In Fig. 2 a typical experiment is presented in which a purified xanthine oxidase preparation was employed. Here oxidation of xanthine proceeds easily if it is present alone with the enzyme. However, this oxidation is strongly inhibited by folic acid. Some oxidation of folic acid appears to occur when it is included in the system alone with the enzyme. This oxidation, however, occurs very quickly and approaches a maximum in about one-half hour. This oxygen uptake might conceivably have arisen from oxidation of aldehydic impurities in the folic acid. However, in this experiment aldehyde-free folic acid was employed. Therefore, this small amount of oxidation appears to arise from oxidation of the folic acid itself, although the reaction is soon inhibited by the product or products formed. Whether the product of the reaction between folic acid and xanthine oxidase is identical with the "activated" folic acid of Jacobson is open to question at this time.

**Summary.** Folic acid whether commercial or aldehyde-free inhibits guanine metabolism and endogenous respiration in rat liver homogenates. Oxidation of folic acid itself in such systems is not clearly evident.

Oxidation of xanthine by a purified preparation of xanthine oxidase is strongly inhibited both by commercial and aldehyde-free folic acid. In a system containing only folic acid and the enzyme some oxidation of the folic acid appears to occur although this reaction is transient.

We are indebted to Dr. D. E. Green of the Institute for Enzyme Research, University of Wisconsin, Madison, for the xanthine oxidase preparation and aldehyde-free folic acid and to the Lederle Laboratories Division, American Cyanamid Company, Pearl River, N. Y., for the folvite. The aldehyde-free folic acid was a gift from the Lederle Laboratories to Dr. D. E. Green.

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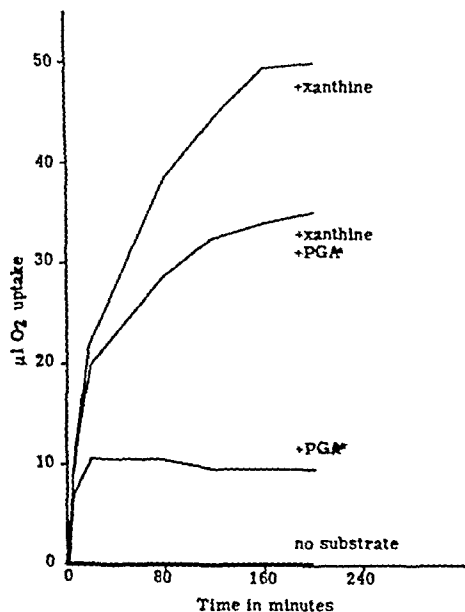


FIG. 2.

Oxygen uptake data showing the interrelationships of xanthine and folic acid in a purified xanthine oxidase preparation.

\* Aldehyde-free. See text.

approximately equimolar amounts of folic acid. However, these workers observed that if folic acid is first incubated alone with the enzyme preparation the characteristic inhibition of xanthopterin oxidation is not observed.<sup>3</sup> They also showed later that this inhibitory effect is given to a much less extent by highly purified folic acid.<sup>4</sup>

Since the structure of the pterin nucleus is very similar to certain of the purines and also to the flavin prosthetic groups, it is possible that inhibition of enzymic reactions by folic acid is competitive in nature. Because of the same structural similarities the possibility also exists that folic acid can be metabolized to some extent by the same reactions. We have studied this problem in relation to both of these possibilities and have concluded that inhibition of purine metabolism *in vitro* by aldehyde-free folic acid does occur. Our results also indicate that folic acid itself may be metabolized to some extent by the same reactions.

**Experimental.** In order to observe the relationship between the *in vitro* metabolism of guanine, the purine most similar in structure to the pterin nucleus and folic acid, oxidation was followed using rat liver hemogenates prepared according to a method of Axelrod and Elvehjem.<sup>5</sup> Livers were obtained from adult, male albino rats maintained on stock ration for several months. The oxidation of guanine, folic acid and guanine plus folic acid was observed for 160 minutes using a conventional Warburg apparatus at 30°C. To each flask was added 1 ml of 16.6% liver homogenate. The levels of substrates employed were as follows: guanine, 0.2 ml 0.02 M; folic acid, 0.2 ml 0.02 M. In addition, 0.2 ml  $1 \times 10^{-4}$  M cytochrome-c and 0.2 ml water were added to each flask. When both guanine and folic acid were included in the same flask, the water was omitted. To absorb carbon dioxide, 0.2 ml 10% KOH was included in the center well of each Warburg

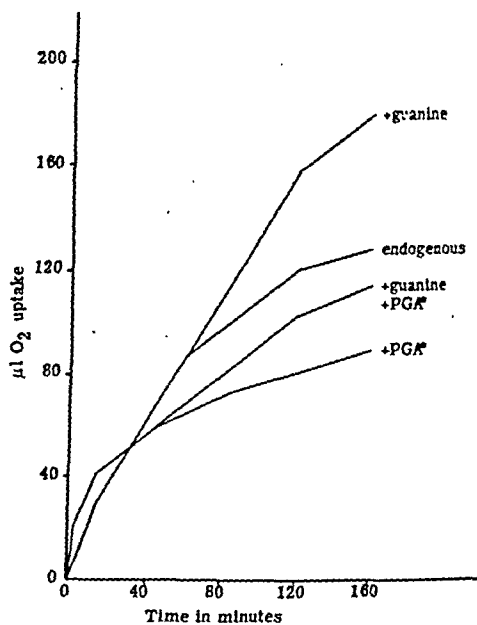


FIG. 1.

Oxygen uptake data showing the influence of folic acid upon guanine metabolism in rat liver homogenate.

\* Aldehyde-free. See text.

vessel.

Since oxidation of xanthopterin by milk xanthopterin oxidase has been shown to be strongly inhibited by samples of folic acid, a purified xanthine oxidase preparation diluted 5-fold with 0.039 M sodium potassium phosphate buffer (pH 7.5) was incubated with xanthine and xanthine plus folic acid (0.2 ml 0.02 M of each substrate). Oxygen uptakes were followed until oxidation of xanthine ceased.

Folic acid concentrations were made equivalent to concentrations of other substrates in respective experiments in order to observe relative oxidation of the substrates. Both commercial folic acid (folvite) and an aldehyde-free preparation of folic acid were employed separately to determine if aldehyde impurities are necessary for the inhibition of xanthine oxidase by folic acid. All substrates were made up in equivalent quantities of alkali.

**Results.** As shown by the curves in Fig. 1, folic acid strongly inhibits endogenous respiration in rat liver *in vitro*. Also metabolism of guanine (presumably via guanase to xan-

<sup>3</sup> Kalekar, H. M., and Klenow, H., *J. Biol. Chem.*, 1948, 172, 351.

<sup>4</sup> Kalekar, H. M., Kjeldgaard, N. O., and Klenow, H., *J. Biol. Chem.*, 1948, 174, 771.

<sup>5</sup> Axelrod, A. E., and Elvehjem, C. A., *J. Biol. Chem.*, 1941, 140, 725.

thine and subsequent oxidation of the xanthine) is decreased about 50%. In the experiment shown aldehyde-free folic acid was employed although almost exactly similar results were obtained when commercial folic acid was employed. Because of the structural similarities between guanine and folic acid it appeared possible that the pterin nucleus could be metabolized similarly to guanine, *i.e.*, a hydrolysis and subsequent oxidation. However, such an effect as this involving oxidation would be obscured by the depression of endogenous respiration by folic acid. It is interesting to note that in the early portion of the curve for flasks containing folic acid there is a brief stimulation of oxygen uptake.

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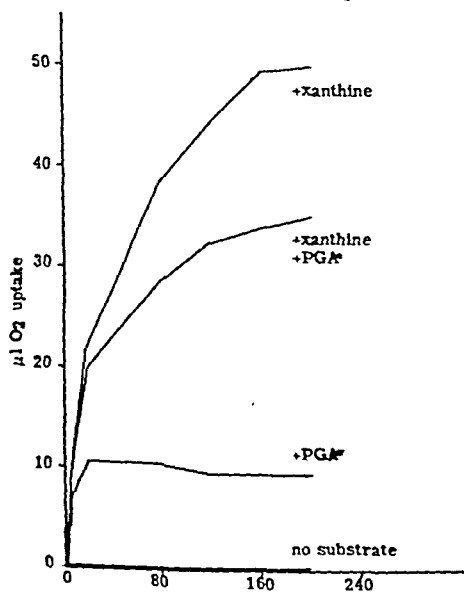


Fig. 2.

Oxygen uptake data showing the interrelationships of xanthine and folic acid in a purified xanthine oxidase preparation.

\* Aldehyde-free. See text.

# 17173. Inhibition of the Antimalarial Activity of Chlorguanide by Pteroylglutamic Acid.

JOSEPH GREENBERG. (Introduced by N. B. Eddy.)

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Chlorguanide (1-(p-chlorophenyl)-5-isopropylbiguanide) is a widely used antimalarial compound, the activity of which was first described by Curd, Davey and Rose.<sup>1</sup> It has been shown to have several interesting relationships with sulfadiazine. When the two drugs are given together there is definite potentiation of effect against *Plasmodium gallinaceum* in the chick.<sup>2</sup> Some chlorguanide-resistant strains are hypersensitive to sulfadiazine,<sup>3</sup> while others are resistant to sulfadiazine.<sup>4</sup> P-aminobenzoic acid will antagonize the antimalarial activity of sulfadiazine<sup>5,6</sup> but not that of chlorguanide.<sup>4</sup> The mode of action of these two drugs is thus not identical but is probably very closely related. It is known that pteroylglutamic acid (PGA) can inhibit the activity of sulfonamides.<sup>7</sup> Experiments were, therefore, undertaken to see if PGA would interfere with the activity of chlorguanide.

**Materials and Methods.** The tests were conducted according to a procedure previously described.<sup>8</sup> New Hampshire Red Chicks, maintained on a commercial starting mash (Purina Startena), were used through-

out. The birds were one week old at the start of an experiment and weighed 42 to 45 g. Treatment was administered twice daily for 4 days beginning 4 to 5 hours before intravenous inoculation with  $16 \times 10^6$  chick erythrocytes parasitized by *P. gallinaceum*. Parasite counts (parasitized erythrocytes per  $10^4$  erythrocytes) were made on the morning of the 4th day after inoculation of parasites (day of inoculation is day 0). A dosage level of drug which causes a 75% reduction in parasitemia in the treated chicks as compared with untreated controls is considered the minimum effective dose. Chlorguanide hydrochloride was administered orally in aqueous solution by catheter and pteroylglutamic acid was administered at the same time and by the same route, in gelatin capsules.

**Experimental.** The minimum effective dose of chlorguanide has been found to be about 0.002 mg/g twice daily for 4 days. Pteroylglutamic acid alone has no significant effect on parasitemia. When the 2 compounds were administered together (Table I), pteroylglutamic acid at 0.002 mg/g did not inhibit the antimalarial activity of chlorguanide. However, PGA at 0.02 mg/g significantly but not completely inhibited the effect of chlorguanide; no further inhibition was produced by 0.2 mg/g. Larger doses of chlorguanide were partially inhibited by PGA.

Pteroylglutamic acid at 0.2 mg/g body weight completely inhibited the minimal effective dose of sulfadiazine, but it had no effect on the antimalarial activity of quinine, chloroquine or pamaquine (Table II).

**Discussion.** The fact that pteroylglutamic acid can inhibit the antimalarial activity of chlorguanide is not proof that chlorguanide is a direct antagonist of PGA. It is possible that the parasite may be able to use PGA to synthesize another metabolite with which

<sup>1</sup> Curd, F. H. S., Davey, D. G., and Rose, F. L., *Ann. Trop. Med.*, 1945, **39**, 208.

<sup>2</sup> Greenberg, J., Boyd, B. L., and Josephson, E. S., *J. Pharm. Exp. Therap.*, 1948, **94**, 60.

<sup>3</sup> Greenberg, J., *J. Nat. Malaria Soc.*, 1949, **8**, 80.

<sup>4</sup> Bishop, A., and McConnachie, E. W., *Nature*, 1948, **162**, 541.

<sup>5</sup> Maier, J., and Riley, E., *Proc. Soc. Exp. Biol. and Med.*, 1942, **50**, 152.

<sup>6</sup> Marshall, E. K., Litchfield, J. T., Jr., and White, H. J., *J. Pharm.*, 1942, **75**, 89.

<sup>7</sup> Lampen, J. O., and Jones, M. J., *J. Biol. Chem.*, 1946, **164**, 485.

<sup>8</sup> Coatney, G. R., and Sebrell, W. H., In F. Y. Wiselogle's "A Survey of Antimalarial Drugs, 1941-1945." J. W. Edwards, Ann Arbor, Mich., 1946.

TABLE I.  
Inhibition by Pteroylglutamic Acid of the Antimalarial Activity of Chlorguanide Against *Plasmodium gallinaceum* in the Chick.

No. chicks	Dose of Chlorguanide HCl (mg/g b.i.d. for 4 days)	Dose of Pteroylglutamic acid (mg/g b.i.d. 4 days)	4th day parasitemia (parasitized erythrocytes per 10 <sup>4</sup> erythrocytes)
Exp. 1			
10	—	—	8070 ± 175
5	.002	—	1674 ± 364
5	.002	.002	990 ± 404
5	.002	.02	3540 ± 558
5	.002	.2	3425 ± 748
Exp. 2			
10	—	—	6840 ± 319
5	.002	—	44 ± 25.1
5	.004	—	44 ± 36.0
5	.008	—	6 ± 1.9
5	.002	.2	5140 ± 276
5	.004	.2	1858 ± 937
5	.008	.2	278 ± 219
Exp. 3			
10	—	—	6790 ± 213
5	.002	—	628 ± 197
5	.002	.02	4520 ± 1010
5	.002	.2	3790 ± 263

TABLE II.  
Effect of Pteroylglutamic Acid on the Antimalarial Activity of Quinine, Pamaquine, Chloroquine and Sulfadiazine. (Five chicks were used in each group).

	Dosage mg/g twice daily for 4 days	Dosage of pteroylglutamic acid mg/g twice daily for 4 days	4th day parasitemia (parasitized erythrocytes/10 <sup>4</sup> erythrocytes)
Untreated	—	—	7244
Quinine hydrochloride	.01	—	6060
	.02	—	942
	.02	.2	796
Chloroquine diphosphate	.001	—	4196
	.002	—	30
	.002	.2	8
Pamaquine citrate	.0005	—	4762
	.001	—	208
	.001	.2	108
Sulfadiazine	.015	—	4120
	.03	—	2170
	.03	.2	7280

chlorguanide directly competes. The fact that it requires about 5 molecules of PGA to inhibit significantly the effect of one molecule of chlorguanide, and that not even 50 molecules of PGA can completely inhibit the effect of one molecule of chlorguanide might indicate that chlorguanide is doing more than competing with PGA. However,

the hypothesis that chlorguanide is a PGA antagonist is an appealing one in view of the relationship between chlorguanide and sulfadiazine, and between the latter and pteroylglutamic acid. In this connection we have found (unpublished data) that 2, 4-diamino-6, 7-diphenylpterin which is believed to stop bacterial growth by preventing the use of

PGA,<sup>9</sup> behaves similarly to chlorguanide in *P. gallinaceum* infections. It potentiates sulfadiazine to about the same degree as chlorguanide and its activity is partially inhibited by the same amounts of PGA as are required to inhibit chlorguanide. If chlorguanide should prove to be a PGA antagonist, this fact would be highly significant in the development of chemotherapeutic agents for malaria and other diseases as well as for

the study of the metabolism of the malaria parasite.

**Summary.** The antimalarial activity of chlorguanide against *Plasmodium gallinaceum* in the chick could be significantly but not completely inhibited by pteroylglutamic acid *in vivo*. Pteroylglutamic acid had no significant effect on the antimalarial activity of quinine, chloroquine or pamaquine, but completely inhibited the effect of sulfadiazine.

<sup>9</sup> Daniel, L. J., and Norris, L. C., *J. Biol. Chem.*, 1947, **170**, 747.

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# 17174. Pancreatic Islet Hyperplasia in Rats Force Fed High Carbohydrate Diets.

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Although both the cat and the dog may develop islet degeneration and irreversible diabetes after prolonged periods of hyperglycemia,<sup>1-6</sup> permanent diabetes has not been produced in the rat by methods short of partial pancreatectomy or alloxan treatment. After noting the ease with which Ingle produced chronic glycosuria in the rat by force feeding a high carbohydrate diet,<sup>7</sup> it occurred to us that it might be of interest to perform a similar experiment and examine the islets of Langerhans.

**Plan of experiments.** Two groups of 6 male albino rats of Sprague-Dawley strain were subjected to force feeding of gradually increasing quantities of high carbohydrate rations until glycosuria was sustained. Weights and urinary glucose excretion were determined at frequent intervals. Rats which died during the

experiment as well those which survived for the entire experimental period of 56 days were examined grossly and histologically, especial attention being given the appearance of the pancreatic islets.

**Rations, method of feeding and urine glucose determinations.** A liquid ration containing about 50% carbohydrate was force fed to each group. The ration fed Group I (Table I) was slightly modified from that described by Ingle.<sup>7</sup> The ration fed Group II was similar except that the carbohydrate was all glucose. The latter diet was employed with the thought that intestinal absorption might be faster and more complete, thus making it possible to obtain higher levels of blood glucose. The rations were force fed 2 times per day at about 8 a.m. and 5 p.m. by means of the technic described by Shay and Gruenstein.<sup>8</sup> Starting with 6 ml per rat the daily portions administered were increased by 4 ml per day until 26 ml were being given. This quantity was maintained for a week to accustom the rats to the method of feeding and to avoid "food-shock".<sup>7</sup> Following this the quantities of ration were gradually in-

<sup>1</sup> Homans, J., *J. Med. Res.*, 1914, **30**, 49.

<sup>2</sup> Homans, J., *J. Med. Res.*, 1915, **33**, 1.

<sup>3</sup> Allen, F. M., *J. Metab. Res.*, 1922, **1**, 75.

<sup>4</sup> Copp, E. F. F., and Barelay, A. J., *J. Metab. Res.*, 1923, **4**, 445.

<sup>5</sup> Dohan, F. C., and Lukens, F. D. W., *Science*, 1947, **105**, 183.

<sup>6</sup> Dohan, F. C., and Lukens, F. D. W., *Endocrinol.*, 1948, **42**, 244.

<sup>7</sup> Ingle, D. J., *Endocrinol.*, 1946, **39**, 43.

<sup>8</sup> Shay, H., and Gruenstein, M., *J. Lab. and Clin. Med.*, 1946, **312**, 1384.

TABLE I.  
Composition of Rations for Force Feeding.

Constituents	Units	Ration I (Group I)	Ration II (Group II)
Egg albumin	g	8	8
Brewers yeast	"	5	5
Corn starch	"	25	—
Dextrin	"	12.5	—
Glucose	"	12.5	50
Ruffex	"	6	6
Salt mixture	"	2	2
Wheat germ oil	"	5	5
Corn oil	"	10	10
Oleum pereomorphum			
Vitamin A	U.S.P. units	200	200
" D	" "	29	29
" K	mg	5	5

Water to make total of 100 ml added to each ration.

creased as tolerated until 68 ml were being given daily. Each group of rats was housed in wire bottomed cages and allowed water *ad libitum*. Beginning on day 7 and on alternate days thereafter the rats of each group were placed in individual metabolism cages and their urine was collected under toluol for a 24-hour period for qualitative and quantitative glucose determinations. Qualitative determinations were performed with 0.5 ml of urine and 5 ml of Benedict's solution which were mixed and heated in a boiling water bath for 5 minutes. Quantitative urine glucose determinations were performed using Benedict's method when the qualitative test showed 2+ or more.

*Histologic preparations.* All tissues were fixed in Bouin's solution and imbedded in paraffin. The sections of pancreas were prepared in Dr. Gomori's laboratory and stained with the chrome hematoxylin and phloxine stain.<sup>9</sup> Hematoxylin and eosin stained sections of liver, stomach, kidney, thymus, and adrenal were examined from all animals and lung, skeletal muscle, mediastinum and testis were studied from selected animals.

*Experimental results.* The quantity of diet administered daily, the average body weights and the urinary glucose values for Group I are summarized in Fig. 1. It is apparent that increasing the diet intake from 26 ml per day to 68 ml per day resulted in a steady increase in body weight amounting to an average

weight gain of almost 200 g for the 8 week period. Urinary glucose became apparent on the 15th day and was excreted by the majority of the animals of this group in quantities varying from 0 to 1.48 g per day for the remainder of the experiment. The animals of this group appeared in moderately good health and only one rat succumbed before the termination of the experiment, due to suffocation from aspirated diet.

The response of the rats of Group II to the ration containing only glucose as the carbohydrate source differed from those of group I, which received the mixed carbohydrate ration in the following respects: the animals tolerated this ration much more poorly and developed signs of food-shock on several occasions, necessitating the interruption of the steady increase of daily rations. Three of the 6 rats succumbed early in the experiment, 2 from food-shock and 1 from false passage of the stomach tube. Two of the remaining 3 rats died before the end of the experiment, 1 of food-shock and 1 of aspiration of diet. The 2 rats which survived beyond the 35th day of the experiment consistently showed more urinary glucose than the rats of Group I. Acetone was noted on 3 occasions in the urine of these rats.

At necropsy the only consistently noted gross change was marked obesity in rats which had been force fed either ration for 30 or more days.

The significant histologic findings in these

<sup>9</sup> Gomori, G., *Am. J. Path.*, 1941, 17, 395.



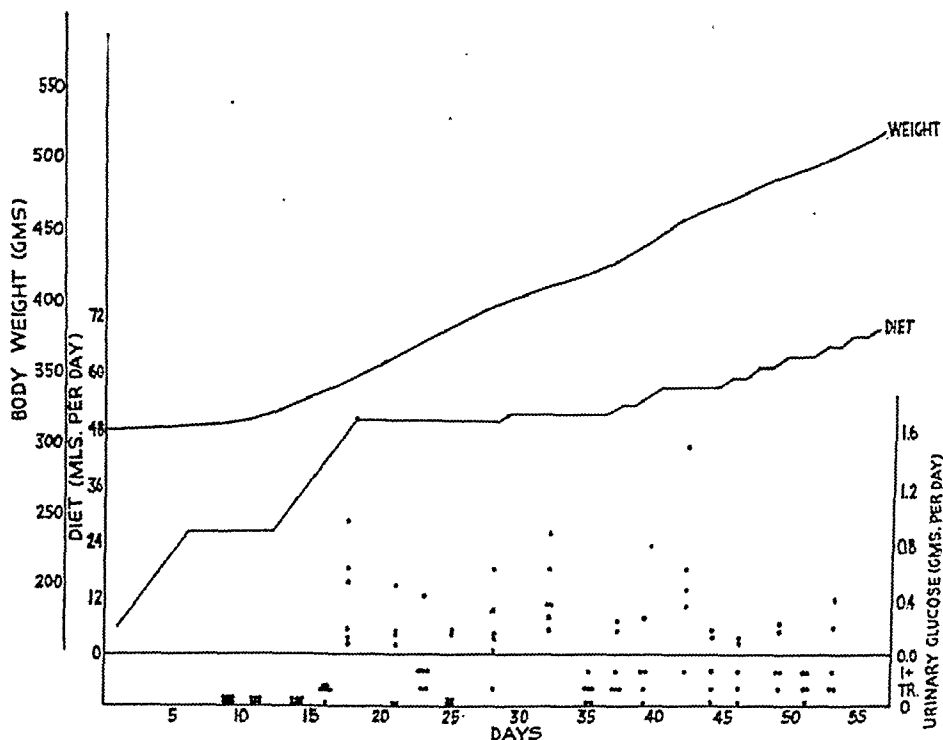


FIG. 1.

Weights, dietary intakes and urinary glucose excretion of rats force fed Diet I.

rats are summarized in Table II. The most striking changes were apparent in the islets of Langerhans. All animals which had received either ration for 32 days or longer showed marked hyperplasia of these structures, accompanied by a consistent decrease in granulation of the beta cells (Fig. 2 and 3). These

changes were well developed in the rats which succumbed at 32 and 38 days and tended to increase as the rations were fed for longer intervals. In most instances the average islet diameters were 2 to 4 times greater than those of control animals. There was no evidence that the rats of Group II showed more marked

TABLE II.  
Histological Findings in Rats Force Fed High Carbohydrate Diets.

Group No.	Rat No.	Time force fed, days	Pancreas		Adrenal cortical hyperplasia	Thymic atrophy	Liver	
			Islet hyperplasia	Degranulation of beta cells			Glycogen	Fat
I	1	55	+++	+++	+	0	+++	+
	2	55	++++	+++	++	0	++++	0
	3	55	++++	+++	+	+	++++	0
	4	55	+++	+++	++++	0	+++	+
	5	38	+++	+++	++	0	+	0
	6	55	++++	+++	0	+	+++	++
II	1	55	+++	+++	++	0	++++	0
	2	14	+	++	0	0	+	0
	3	10	0	+	0	0	++	0
	4	14	+	++	+++	0	+	+
	5	32	++	+++	0	+	+	0
	6	51	+++	+++	0	0	++++	0

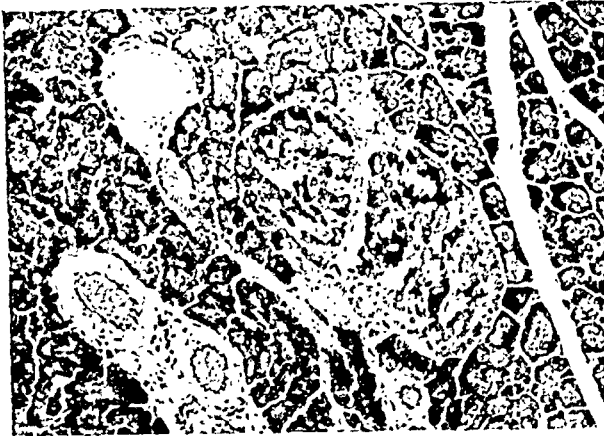


FIG. 2.  
Typical pancreatic islet from rat II-3 which died after only 10 days of force feeding. Note the almost normal appearance of the islet cells. Chrome-hematoxylin and phloxine stain.  $\times 150$ .

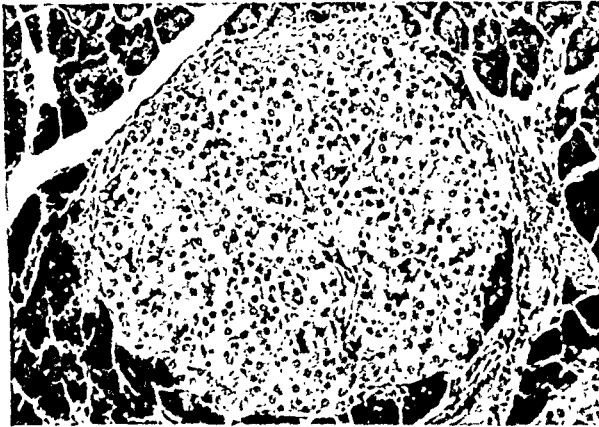


FIG. 3.  
Typical pancreatic islet from rat II-1 which was sacrificed after 56 days of force feeding. Note the marked hyperplasia of the islet cells with almost complete degranulation of the beta cells. Chrome hematoxylin and phloxine stain.  $\times 150$ .

changes. Although the pitfalls of evaluating islet volume from examination of a single section through the pancreas are well established,<sup>10</sup> these hyperplastic changes were so uniform and conspicuous that there is little doubt of their significance. Furthermore, the recent study of Tejning<sup>11</sup> gives us added confidence in the validity of our interpretation.

<sup>10</sup> Richardson, K. C., and Young, F. G., *J. Physiol.*, 1937, 91, 352.

After detailed studies of pancreatic islet volumes, this investigator concluded that if one finds in a section of pancreas a number of islets that are larger than normal, one can conclude that the total volume of islet tissue is also greater than normal. No evidence of necrosis, hydropic change, hemorrhage, inflammation or absolute decrease in beta cells

<sup>11</sup> Tejning, S., *Acta Med. Scandinav. Supp.*, 198, 1947.

was apparent in any of the sections.

Other significant histologic findings consisted of a somewhat variable but often marked increase in glycogen in the liver cord cells, sometimes accompanied by slight large droplet fatty change. The stomachs of the rats which had been force fed for a period of 30 days or more showed a spectacular hypertrophy of the muscularis but no other abnormality. The thymus glands showed microscopic evidence of atrophy in only 3 rats, and this was in no instance well developed. The other organ showing rather consistent alteration was the adrenal. Here there was a moderate cortical hyperplasia which was evident in 7 rats. There was no apparent correlation between the adrenal cortical hyperplasia and the thymic atrophy.

The kidneys as well as the skeletal muscle and the structures of the mediastinum revealed no significant histologic changes in the instances in which they were examined. Unfortunately, the testes were studied microscopically in only one rat (number 4, Group I). Here the tubules were of normal size and cellularity and spermatogenesis was apparently active.

*Discussion.* Our data are not complete enough to warrant conclusions to be drawn regarding the adrenal cortical hyperplasia. The similarly treated rats of Ingle<sup>7</sup> uniformly showed grossly enlarged, dark red, adrenal cortices. The findings may be due to the very large amounts of carbohydrate administered, to the stress incident to the acts of force feeding, or to some other factor. High protein diets have been reported to cause adrenal cortical enlargement,<sup>12</sup> but the protein contained in the diets fed to our rats probably was not sufficient at any time to cause such changes.

The concentration of sugar in the blood has repeatedly been found to affect islet structure and volume. The islet cell degeneration produced by partial pancreatectomy and/or anterior pituitary extract injections in the dog and cat can be prevented or, in certain instances, reversed by a low carbohydrate

intake, insulin, or phloridzin.<sup>1-4,13</sup> Rabbits treated with alloxan develop hydropic degeneration of the islets after about one month of marked hyperglycemia.<sup>14</sup> Corpaci believed that he had increased islet volume and produced damage of islet cells in rabbits by repeated parenteral injections of glucose and adrenalin, but the evidence is not conclusive.<sup>15</sup> Similar experiments in guinea pigs by Meneghini, using glucose alone, are reported to have caused islet hyperplasia.<sup>16</sup> Careful studies have shown that rats fed a high carbohydrate diet *ad libitum* develop pancreatic islet volumes that are significantly greater than those fed *ad libitum* other types of diets.<sup>11</sup> Evidence has been obtained of both hyperplasia and exhaustion of guinea pig islets after continuous infusions of large amounts of glucose.<sup>17,18</sup> In experiments lasting not more than 100 hours, dogs killed by the continuous intravenous administration of glucose were found to have specific intense hemorrhages into and destruction of the pancreas and hypophysis.<sup>19</sup> Special studies of islets were not reported. Finally, clinical diabetes has been produced in an intact cat after 39 days of intraperitoneal glucose injections, and part of a series of cats treated in this way developed marked hydropic degeneration of the islets.<sup>5,6</sup> Verzar and von Kúthy produced heavy glycosuria, mild hyperglycemia, and, on rare occasions, ketonuria in dogs by force feeding solutions of glucose and sucrose for several weeks.<sup>20</sup> Glucose tolerance was thereby impaired but soon returned to normal. Post-mortem examinations are not recorded.

Degeneration of rat pancreatic islets has not been produced by hyperglycemia. Lukens

<sup>13</sup> Lukens, F. D. W., Dohan, F. C., and Wolcott, M. W., *Endocrinol.*, 1943, **32**, 475.

<sup>14</sup> Duff, G. L., McMillan, G. C., and Wilson, D. C., *Proc. Soc. Exp. Biol. and Med.*, 1947, **64**, 251.

<sup>15</sup> Corpaci, A., *Sperimentale, Arch. di Biol.*, 1932, **86**, 120.

<sup>16</sup> Meneghini, T., *Ginecologia*, 1939, **5**, 539.

<sup>17</sup> Woerner, C. A., *Anat. Rec.*, 1938, **71**, 33.

<sup>18</sup> Woerner, C. A., *Anat. Rec.*, 1939, **75**, 91.

<sup>19</sup> Jacobs, H. R., and Colwell, A. R., *Am. J. Physiol.*, 1936, **116**, 104.

<sup>20</sup> Verzar, F., and von Kúthy, A., *Pflüger's Arch. f. d. ges. Physiol.*, 1930, **225**, 606.

<sup>12</sup> Tepperman, J., Engel, F. L., and Long, C. N. H., *Endocrinol.*, 1943, **32**, 403.

and Dohan examined the pancreatic remnants of rats that had had pronounced hyperglycemia for from 2 weeks to several months following partial pancreatectomy and found almost no damage.<sup>21</sup> In similarly treated animals hyperplasia instead of damage was reported by Friedman and Marble.<sup>22</sup> The same type of change was observed after the administration of anterior pituitary extract.<sup>10</sup> In order to produce glycosuria consistently in the rat with anterior pituitary extract, partial pancreatectomy is necessary,<sup>23</sup> although adrenal steroids cause temporary insulin resistant diabetes.<sup>24</sup> However, Peterson has recently reported complete degranulation of rat islet beta cells within 15 minutes of the intracardiac injection of 3.0 g of glucose/kg.<sup>25</sup> This lasts 48 hours and is associated with temporary impairment of glucose tolerance. Similar but less consistent degranulation was found after one intraperitoneal injection of

glucose in the guinea pig by Gomori *et al.*<sup>26</sup>

In canine anterior pituitary diabetes, proliferation of the islet cells may precede the exhaustive or degenerative changes which accompany the onset of permanent diabetes.<sup>27</sup> Our observations indicate that the carbohydrate tolerance increased with each increase in feeding, glycosuria decreasing when the diet was maintained at a level which had initially caused moderate sugar excretion. This adaptation is evidently implemented by islet hyperplasia. Although it might be possible to prevent hyperplasia and produce islet damage by increasing the volume of food more rapidly this would probably be difficult without causing fatal "food-shock." It is conceivable that some relatively small additional stimulus, perhaps the administration of anterior pituitary extract or adrenal steroids, might have produced exhaustion of the islets of our rats. Older rats may be more susceptible.<sup>28</sup>

**Summary.** Degranulation of beta cells of the pancreatic islets and islet hyperplasia have been observed in rats which were force-fed large amounts of high carbohydrate, otherwise balanced diets for periods of as long as 8 weeks. Glycosuria and, in rare instances, ketonuria occurred.

<sup>21</sup> Lukens, F. D. W., and Dohan, F. C., *Endocrinol.*, 1942, **30**, 175.

<sup>22</sup> Friedman, N. B., and Marble, A., *Endocrinol.*, 1944, **29**, 577.

<sup>23</sup> Long, C. N. H., *Harvey Lect.*, 1936-37, **32**, 194.

<sup>24</sup> Ingle, D. J., Sheppard, R., Evans, J. S., and Kuizenga, M. H., *Endocrinol.*, 1945, **37**, 341.

<sup>25</sup> Peterson, C. A., *Proc. Soc. Exp. Biol. and Med.*, 1949, **70**, 352.

<sup>26</sup> Gomori, G., Friedman, N. B., and Caldwell, D. W., *Proc. Soc. Exp. Biol. and Med.*, 1939, **41**, 567.

<sup>27</sup> Richardson, K. C., and Young, F. G., *Lancet*, 1938, **1**, 1098.

<sup>28</sup> Martinez, C., *Rev. Asoc. med. argent.*, 1946, **60**, 686.

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## 17175. Inorganic Aging of the Plasma Layer of Tissue Cultures.

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The problem of maintaining mammalian cells *in vitro* for long intervals in an original or lightly patched plasma base may be broken down into two aspects: (a) the avoidance of calcification during the usual transfer intervals of two to four weeks and (b) the problem of an inorganic aging which complicates

more prolonged maintenance. Though the control of calcification difficulties during the usual transfer intervals seems feasible by the measures summarized in the preceding paper,<sup>1</sup> solution of the long-term problems

<sup>1</sup> Hanks, J. H., *Proc. Soc. Exp. Biol. and Med.*, 1949, **71**, 328.

was apparent in any of the sections.

Other significant histologic findings consisted of a somewhat variable but often marked increase in glycogen in the liver cord cells, sometimes accompanied by slight large droplet fatty change. The stomachs of the rats which had been force fed for a period of 30 days or more showed a spectacular hypertrophy of the muscularis but no other abnormality. The thymus glands showed microscopic evidence of atrophy in only 3 rats, and this was in no instance well developed. The other organ showing rather consistent alteration was the adrenal. Here there was a moderate cortical hyperplasia which was evident in 7 rats. There was no apparent correlation between the adrenal cortical hyperplasia and the thymic atrophy.

The kidneys as well as the skeletal muscle and the structures of the mediastinum revealed no significant histologic changes in the instances in which they were examined. Unfortunately, the testes were studied microscopically in only one rat (number 4, Group I). Here the tubules were of normal size and cellularity and spermatogenesis was apparently active.

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The concentration of sugar in the blood has repeatedly been found to affect islet structure and volume. The islet cell degeneration produced by partial pancreatectomy and/or anterior pituitary extract injections in the dog and cat can be prevented or, in certain instances, reversed by a low carbohydrate

intake, insulin, or phloridzin.<sup>1-4,13</sup> Rabbits treated with alloxan develop hydropic degeneration of the islets after about one month of marked hyperglycemia.<sup>14</sup> Corpaci believed that he had increased islet volume and produced damage of islet cells in rabbits by repeated parenteral injections of glucose and adrenalin, but the evidence is not conclusive.<sup>15</sup> Similar experiments in guinea pigs by Meneghini, using glucose alone, are reported to have caused islet hyperplasia.<sup>16</sup> Careful studies have shown that rats fed a high carbohydrate diet *ad libitum* develop pancreatic islet volumes that are significantly greater than those fed *ad libitum* other types of diets.<sup>11</sup> Evidence has been obtained of both hyperplasia and exhaustion of guinea pig islets after continuous infusions of large amounts of glucose.<sup>17,18</sup> In experiments lasting not more than 100 hours, dogs killed by the continuous intravenous administration of glucose were found to have specific intense hemorrhages into and destruction of the pancreas and hypophysis.<sup>19</sup> Special studies of islets were not reported. Finally, clinical diabetes has been produced in an intact cat after 39 days of intraperitoneal glucose injections, and part of a series of cats treated in this way developed marked hydropic degeneration of the islets.<sup>5,6</sup> Verzar and von Kúthy produced heavy glycosuria, mild hyperglycemia, and, on rare occasions, ketonuria in dogs by force feeding solutions of glucose and sucrose for several weeks.<sup>20</sup> Glucose tolerance was thereby impaired but soon returned to normal. Post-mortem examinations are not recorded.

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<sup>14</sup> Duff, G. L., McMillan, G. C., and Wilson, D. C., *Proc. Soc. Exp. Biol. and Med.*, 1947, **64**, 251.

<sup>15</sup> Corpaci, A., *Sperimentale, Arch. di Biol.*, 1932, **86**, 129.

<sup>16</sup> Meneghini, T., *Ginecologia*, 1939, **5**, 539.

<sup>17</sup> Woerner, C. A., *Anat. Rec.*, 1938, **71**, 33.

<sup>18</sup> Woerner, C. A., *Anat. Rec.*, 1939, **75**, 91.

<sup>19</sup> Jacobs, H. R., and Colwell, A. R., *Am. J. Physiol.*, 1936, **116**, 194.

<sup>20</sup> Verzar, F., and von Kúthy, A., *Pflueger's Arch. f. d. ges. Physiol.*, 1930, **225**, 606.

<sup>12</sup> Tepperman, J., Engel, F. L., and Long, C. N. H., *Endocrinol.*, 1943, **32**, 403.

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*Summary.* Degranulation of beta cells of the pancreatic islets and islet hyperplasia have been observed in rats which were force-fed large amounts of high carbohydrate, otherwise balanced diets for periods of as long as 8 weeks. Glycosuria and, in rare instances, ketonuria occurred.

<sup>21</sup> Lukens, F. D. W., and Dohan, F. C., *Endocrinol.*, 1942, **30**, 175.

<sup>22</sup> Friedman, N. B., and Marble, A., *Endocrinol.*, 1944, **29**, 577.

<sup>23</sup> Long, C. N. H., *Harvey Lect.*, 1936-37, **32**, 194.

<sup>24</sup> Ingle, D. J., Sheppard, R., Evans, J. S., and Kuizenga, M. H., *Endocrinol.*, 1945, **37**, 341.

<sup>25</sup> Peterson, C. A., *Proc. Soc. Exp. Biol. and Med.*, 1949, **70**, 352.

<sup>26</sup> Gomori, G., Friedman, N. B., and Caldwell, D. W., *Proc. Soc. Exp. Biol. and Med.*, 1939, **41**, 567.

<sup>27</sup> Richardson, K. C., and Young, F. G., *Lancet*, 1938, **1**, 1098.

<sup>28</sup> Martinez, C., *Rev. Asoc. med. argent.*, 1946, **60**, 666.

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## 17175. Inorganic Aging of the Plasma Layer of Tissue Cultures.

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The problem of maintaining mammalian cells *in vitro* for long intervals in an original or lightly patched plasma base may be broken down into two aspects: (a) the avoidance of calcification during the usual transfer intervals of two to four weeks and (b) the problem of an inorganic aging which complicates

more prolonged maintenance. Though the control of calcification difficulties during the usual transfer intervals seems feasible by the measures summarized in the preceding paper,<sup>1</sup> solution of the long-term problems

<sup>1</sup> Hanks, J. H., *Proc. Soc. Exp. Biol. and Med.*, 1949, **71**, 328.

arising from inorganic aging appears more formidable.

It is a classical observation that even in the absence of evident calcification the plasma layer of cell cultures gradually becomes less and less favorable to cell growth. Simms and Stillman<sup>2</sup> have presented evidence that tryptic digestion removes from adult tissues and from the plasma of cell cultures an inhibitor having some of the properties of calcium phosphate. It is also known that inorganic gels undergo changes in viscosity, pH, extinction coefficients, and other physical properties during aging. These changes can be explained in part by preferential liberation and adsorption of ions, that is by ionic exchange.<sup>3</sup> Particularly in circumstances where a gel is in contact with supernatant fluids which are changed repeatedly, the concentration of preferentially adsorbed ions will become radically different from the ionic analysis at the moment the gel is formed.<sup>4</sup> Such procedures are an inherent feature of the long-term tissue culture techniques.

It is the purpose of the present communication to present evidence concerning the adsorption of calcium by the plasma layer of tissue cultures and the difficulty of decalcifying this substrate. Methods have been summarized in the preceding paper.<sup>1</sup>

*Experimental observations.* The firmness with which calcium and phosphates are adsorbed to the plasma layer became apparent as the result of attempts to remove opacity from cell colonies and plasma which had become calcified. This opacity could not be diminished by using supernatant media prepared by diluting the serum and embryo juice in salt solution lacking both Ca and P or by prolonged depression of pH during cultivation within physiological limits of H ion concentration. Incorporation of 0.1 to 0.2% sodium citrate in media of minimal Ca and P levels or in mildly acidified washing solutions killed the cells without clearing the plasma. Direct extraction with N/100 HCl in the

balanced salt solution or weaker acids in the balanced salt solution likewise failed to clear the plasma before the cells were irreparably damaged. The deposits were rapidly removed by N/10 HCl, which was fatal to cells. Optimal cell survival followed rapid clearing of the plasma in N/40 HCl in the salt solution for 5 to 20 minutes as required. Since the complicated procedure required to effect this result and obtain prompt neutralization without cell damage is not to be recommended,\* these observations are summarized only in order to illustrate the fact that the Ca adsorbed by the plasma is not brought into solution by complex-forming anions such as citrate and is liberated only by ionic exchange with fairly high concentrations of H ion.

Other evidence of the role of ionic exchange in the inorganic aging of plasma was obtained from data on the increased deposition of Ca in the plasma layer during repeated renewal of cultures with supernatant fluids which do not produce calcification within the usual transfer intervals. After 6 weeks of cultivation in media composed of human serum 40% and beef embryo juice 4% diluted in the salt solution containing Ca 5 mg% and P 2.2 mg% (six renewals) the slightly opalescent plasma layers of 10 cultures were rinsed and extracted with N/10 HCl. Analysis revealed that this plasma contained from 24 to 40 mg% of Ca, i.e., three to five times the original concentration of Ca in the plasma.

When the same formula (sera 40%, embryo

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\* A more practical procedure is the complete removal of opaque plasma by digestion with 0.5% trypsin or pancreatin in the salt solution at pH 7.8. This process must be watched carefully and stopped before the naked cells, which retract around the explants, are washed away (all liberated fibroblasts appear to be damaged). Since digestion is never completed simultaneously in all cultures, the simplest procedure is to replace the digestion mixture in each culture with two drops of 50% plasma at the appropriate moment, to exhale alveolar air to depress the pH, and to stopper quickly. The liquid plasma should be rolled around the culture vessels several times and then be allowed to cover the explants and establish a bond with the wet glass before the third drop of medium or coagulant is added to induce simultaneous coagulation in all cultures thus accumulated.

<sup>2</sup> Simms, H. S., and Stillman, N. P., *J. Gen. Physiol.*, 1937, **20**, 603.

<sup>3</sup> Hanks, J. H., and Weintraub, R. L., *J. Phys. Chem.*, 1937, **41**, 583.

<sup>4</sup> Jenny, H., *J. Phys. Chem.*, 1932, **36**, 2217.

juice 4%) is prepared in salt solution lacking both Ca and P, it contains approximately one half the usual levels of these elements. Though this solution delays the darkening of the central portion of transplants, there is no deficit in calcium compounds, and the central transplants eventually become opaque to transmitted light. This solution is superior to the usual formula for prolonged maintenance of cells in a plasma base.

*Discussion.* The demonstration of a 5-fold increase of Ca ions in a plasma base following serial renewals of a supernatant fluid, and of the necessity of hydrogen ions for its replacement, indicates that the plasma substrate in cell cultures is the site of an ionic exchange. A more complete analysis could be expected to show that not only Ca but also other preferentially adsorbed ions tend to replace less tightly bound but physiologically important ions. It may be inferred that cells maintained in an original or lightly patched plasma base spend a considerable portion of their existence in an inorganic environment quite different from what is intended and that the inorganic composition of renewal super-

nates might be improved by altering radically the ionic balance now normally present in fluids prepared from complex nutrients. It is also evident that existing data on the role of different ions in plasma cultures may require re-evaluation by culture methods which expose the cells directly to the fluids being studied.

The present data would appear to confirm the observations of Simms and Stillman<sup>2</sup> on the nature of at least one of the inhibitors which occur in plasma and adult tissues and is capable of being removed by tryptic digestion.

*Summary.* The adsorption of calcium phosphate complexes to plasma substrates, and the conditions required for their removal, indicate that the plasma gel is the site of an ionic exchange in which preferentially adsorbed ions may greatly alter the desired ionic balance. This process is one of the factors which causes plasma to become growth inhibitory during prolonged maintenance of cells in this type of substrate.

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## 17176. Tolerance of Normal and Partially Depancreatized Rats for Insulin.

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It was shown by this study that the average lethal dose of insulin is smaller in force-fed depancreatized rats than in normal rats.

*Methods.* Male rats of the Sprague-Dawley strain were maintained on Archer Dog Pellets prior to the beginning of the experiments. At a weight of 310-320 g. some of the animals were subjected to a single-stage pancreatectomy<sup>1</sup> in which all but tiny remnants lying between the duodenum and bile duct were removed. After the rats recovered from the operation, they were placed in metabolism

cages and were adapted to the force-feeding<sup>2</sup> of a medium carbohydrate diet (Table I) in amounts of 13 cc each morning and late afternoon. The urines were collected at 8:00 to 8:30 A.M. each day and were preserved by thymol. Urinary glucose was determined by the method of Benedict.<sup>3</sup>

*Experiments and results.* Severely diabetic rats were selected for these experiments. The average amount of glucose excreted by the 18 depancreatized rats used in this study was 6096 mg per day. The amount of glucose es-

<sup>1</sup> Ingle, D. J., and Griffiths, J. Q., Jr., Chapter 16, *The rat in laboratory investigation*, J. B. Lippincott Co., Philadelphia, 1942.

<sup>2</sup> Reinecke, R. M., Ball, H. A., and Samuels, L. T., *Proc. Soc. Exp. Biol. and Med.*, 1939, 41, 44.

<sup>3</sup> Benedict, S. R., *J.A.M.A.*, 1911, 57, 1193.



TABLE I.  
Medium Carbohydrate Diet.

Constituent	g
Cellu flour (Chicago Dietetic Supply)	120
Osborne & Mendel salt mixture	40
Dried yeast (Pabst)	100
Wheat germ oil	10
Cod liver oil	10
Vit. K (2-methyl-1,4-naphthoquinone)	100 mg
Mazola oil	200
Casein (Labco)	160
Starch	200
Dextrin	190
Sucrose	200
Water to make total of	2000 cc

TABLE II.  
Lethal Dose of Regular Insulin Expressed as Units  
for Rat per Day. Means and standard deviations  
of the means and differences.

	No.	Mean	Difference
Normal	20	68.5 $\pm$ 1.77	
Depancreatized	18	50.5 $\pm$ 4.27	18 $\pm$ 4.68

timated to be available from the carbohydrate, glycerol and protein (assuming 60% conversion of protein to glucose) of the diet is approximately 6500 mg per rat per day. These severely diabetic rats were then compared to normal force-fed rats in respect to the amount of insulin required to kill the animal. Regular insulin (Lilly) was administered by subcutaneous injection immediately following each feeding. The initial dose was 5 units given twice each day. The dosage was increased by 5 units per injection on every second day until the animal died.

The data are summarized in Table II. Normal rats tolerated greater average amounts of insulin than did the diabetic rats. The variability among individual animals was greater among the depancreatized rats than among the normal animals. The difference between the means for the two groups is highly significant from the statistical standpoint.

**Discussion.** If it were assumed that the secretion of insulin is unaltered by the administration of exogenous insulin, it would be expected that a lethal level of insulin dosage would be reached more rapidly in the normal animal than in the depancreatized animal. It seems probable from these and other data<sup>4</sup>

that the secretion of insulin by the pancreatic islets is inhibited by the administration of an excess of exogenous insulin. Even this assumption does not explain the increased sensitivity of the depancreatized animal to insulin. It has been noted that totally depancreatized patients<sup>5</sup> may show greater sensitivity to insulin than the average patient with diabetes mellitus. Young<sup>6</sup> reported that pancreatectomy of a dog with pituitary diabetes lowered its insulin requirement. Dragstedt *et al.*<sup>7</sup> noted that partially depancreatized dogs may have a higher insulin requirement than totally depancreatized dogs. Thorogood and Zimmerman<sup>8</sup> found that dogs having alloxan diabetes showed a decrease in insulin requirement following pancreatectomy. Howard<sup>9</sup> has commented upon "The almost uniform finding that an organism is highly oversensitive to a hormone that it lacks, and the corollary that the normal is relatively insensitive to doses of a hormone which would seriously injure the deficient animal. . . one wonders if the gland itself may not sometimes reverse its processes, and, in the presence of an administered excess of its hormone, actually begin to destroy it or elaborate some counteracting hormone." A substance which causes glycogenolysis and hyperglycemia has been obtained from extracts of pancreatic islets<sup>10</sup> and has been considered as a possible antagonist of insulin but it is not known if it is a normal secretory product of the pancreas or if it has any physiological role.

An additional explanation of these results could be based upon the assumption that the

<sup>5</sup> Waugh, J. M., Dixon, C. F., Clagett, O. T., Bollman, J. L., Sprague, R. G., and Comfort, M. W., *Proc. Staff. Meet. of the Mayo Clinic*, 1946, **21**, 25.

<sup>6</sup> Young, F. G., *New England J. Med.*, 1939, **221**, 635.

<sup>7</sup> Dragstedt, L. R., Allen, J. G., and Smith, E. M., *Proc. Soc. Exp. Biol. and Med.*, 1943, **54**, 292.

<sup>8</sup> Thorogood, E., and Zimmerman, B., *Endocrinology*, 1945, **37**, 191.

<sup>9</sup> Howard, J. E., *American Practitioner*, 1948, **2**, 674.

<sup>10</sup> Heard, R. D. H., Lozinski, E., Stewart, L., and Stewart, R. D., *J. Biol. Chem.*, 1948, **172**, 857.

<sup>4</sup> Haist, R. E., *Physiol. Rev.*, 1944, **24**, 409.

assimilation of food was incomplete due to a deficiency in the external secretion of the pancreas and that fatal hypoglycemia developed more rapidly for this reason. There was no diarrhea in these experiments, the feces were well formed and firm but were larger than normal in the depancreatized rats. The completeness of intestinal absorption was not studied. Similarly, it is possible that the depancreatized rats were debilitated by operation and by the period of uncontrolled glycosuria so that their resistance to insulin shock was lower than normal. Although the depancreatized animals appeared vigorous and healthy during the period of insulin administration, it would be unsafe to conclude that their lower than normal resistance to insulin

was due solely to the absence of the pancreatic islets.

*Summary.* Eighteen extensively depancreatized male rats and 20 normal rats were forced a medium carbohydrate diet. Following periods of adaptation and control, all of the animals were tested for their resistance to twice daily injections of regular insulin. The initial dose was 5 units and was increased in increments of 5 units per dose every second day until the animal died. The average lethal dose for the depancreatized rats was 50.5 units per 24 hours as compared to an average of 68.5 units for the normal animals. This difference in averages was calculated to be statistically significant.

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### 17177. Isolation of Three Strains of Type B Influenza Virus Incompletely Related to Lee.

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During December 1948, reports of widespread distribution of influenza began to appear in the Seattle area. The majority of illnesses were of relatively short duration and were accompanied in many cases by gastrointestinal disturbances. Proper specimens of throat washings and serum were not obtainable until the return of students to the University of Washington campus after the New Year holidays.

During the first 9 weeks of 1949, there were 116 admissions for upper respiratory infection to the University of Washington Health Center. It was apparent, through random questioning of the campus population, that many others were ill at home. Since approximately 60% of the 16,000 University students live at home in or close to the City of Seattle, and there were a number of students known to be ill in living quarters on and off the campus, it seems obvious that the outbreak was much more widespread than the 116 admissions to the Health Center would

indicate. A conservative estimate of 500 cases, of varying severity, during the period from the end of December to the first of March does not seem unreasonable. Observations of classroom attendance supported this estimate.

The majority of illnesses were mild, with the average period of hospitalization being between 3 and 4 days. The mildness of the disease further supports the idea that the cases seen in the campus infirmary represented only a relatively small proportion of the total number occurring in the Seattle area.

*Materials.* Blood specimens and throat washings were obtained during the acute stage from patients in the infirmary whenever possible. Serum was separated promptly and stored at  $-2^{\circ}\text{C}$  until used. Throat washings were obtained by having the subjects gargle 15 ml of nutrient broth (Bacto) to which had been added penicillin G in an approximate concentration of 1000 units per ml. The garglings were collected in clean beakers, trans-

ferred to sterile screw cap bottles, and stored in a CO<sub>2</sub> ice box at approximately -76°C until used.

Convalescent blood specimens were obtained whenever possible. The interval between the acute and convalescent specimens ranged from 10 to 25 days, with a few exceptions. Twenty-eight convalescent sera were obtained from suspected influenza cases, and were stored with the acute specimens at -2°C.

**Methods.** Throat washings were inoculated to the allantoic cavity of 8- to 10-day chick embryos, using 0.25 ml per egg and a minimum of 5 eggs per washing. Inoculated eggs were incubated at 36°C and were examined daily by candling. Eggs showing dead embryos in 24 hours were discarded. A small portion of allantoic fluid was aspirated daily from surviving eggs from the second to fifth days after inoculation, and was tested in a 1 to 10 dilution for ability to agglutinate washed human cells of group O. Fluids showing typical agglutination were inoculated to a new set of eggs and were further tested against standard antisera in an attempt to determine the type of virus most prevalent in the outbreak.

The technic for examination of paired sera was that recommended by the Influenza Information Center of the U. S. Public Health Service<sup>1</sup> for laboratories participating in its program. The original method,<sup>2</sup> as modified by the Army Medical Center,<sup>3</sup> involves the addition of 4 hemagglutinating units of the standard virus antigens (PR8, FM1 and Lee) to serial two-fold dilutions of inactivated serum, followed by addition of washed group O human red cells. Incubation was for one hour at room temperature.

**Results.** Three strains of influenza virus were isolated during the early part of the outbreak, and gave typical agglutination results up to 1 in 320, in the first passage. Subsequent allantoic passages yielded fluids

with titers ranging up to 1 in 2560. The strains were designated as Seattle 1-49, 2-49, and 3-49, and were forwarded to the Influenza Strain Study Center.<sup>4</sup>

The 3 virus strains were separately tested by the standard agglutination-inhibition technic against high-titer monovalent rooster sera.\* These sera were able to inhibit red cell agglutination by PR8, FM1 or Lee antigens in titers of 1 in 1600 or 1 in 800, with cross-inhibition only in the first tube between the A and A prime viruses. However, when the 3 newly isolated strains of influenza virus were set up against the standard sera, no significant evidence of inhibition of hemagglutination could be obtained. Additional egg passages up to a total of 10 did not modify these results. The specific rooster sera were not able to supply the typing information desired, and because of doubt regarding the nature of the prevalent virus, a general program of vaccination was not undertaken.

Twenty-eight paired sera were investigated for evidence of rise in antibody titer against types A, A prime and B viruses, and also against the Seattle strains, using the standard agglutination-inhibition technic. No pairs showed any evidence of significant increase against type A or A prime viruses and these data are therefore omitted. The results of the significant titrations are shown in Tables I and II.

Table I gives the results of titrations on 28 paired sera. Six patients showed no increase of antibodies able to inhibit hemagglutination by known virus antigens and were not considered as serologically proved cases. The titrations of the 22 sera showing a significant increase of such antibodies gave the results indicated. It will be noted that the evidence indicates a higher antibody rise against Lee virus than against the Seattle strains.

The difference in antigenicity between the Lee and Seattle viruses is demonstrated further in Table II. It is seen that of the 22 serum pairs showing a 4-fold or greater increase in titer against Lee virus, only 18 also showed

<sup>1</sup> Culbertson, James T., *Am. J. Pub. Hlth.*, 1949, 39, 37.

<sup>2</sup> Anonymous, *Bull. U. S. Army Med. Dept.*, 1946, 6, 777.

<sup>3</sup> Medical Division, Army Medical Center, letter of Sept. 7, 1948.

<sup>4</sup> Anonymous, *Science*, 1947, 106, 542.

\* Obtained through the kindness of the Department of Virus and Rickettsial Diseases, Army Medical Center, Washington, D.C.

TABLE I.  
Titrations of Paired Sera.

Increase	4-fold	8-fold	16-fold or more	Total
vs. Lee	6	7	9	22
vs. Seattle strains	9	8	1	18
No significant increase in titer				6

TABLE II.  
Increase in Titer of Paired Sera.

Significant increase	
vs. both Lee and Seattle strains	18
vs. Lee only	4
vs. Seattle strains only	0
No increase	6
Total	28

a significant rise against the Seattle strains. Four patients showing a rise in titer of antibodies able to inhibit hemagglutination by Lee virus and not by the Seattle strains, may represent cases due to viruses other than the more prevalent strains.

On the basis of titrations of paired sera only, the outbreak in Seattle would be considered as due to a virus antigenically related to Lee virus, since all the positive sera showed a significant increase in antibodies against Lee when compared with the acute phase sera. On the other hand, hemagglutination by the Seattle viruses was not inhibited by typing sera prepared in roosters. This fact emphasizes two points: first, an antigenic difference between the newly isolated strains and Lee virus, and second, the inadequacy of highly specific antisera for the typing of influenza virus having minor antigenic differences from the standard strains.

Because of the ability of Seattle viruses to produce antibodies against Lee virus, and the failure of specific Lee antiserum to inhibit hemagglutination by the new strains, it is apparent that the Seattle strains are not antigenically identical with Lee virus. This opin-

ion has been confirmed by the virus laboratory of the California State Department of Health<sup>5</sup> and by the Influenza Strain Study Center.<sup>6</sup>

The evidence in the case of the Seattle strains seems to indicate that these viruses are not as highly antigenic as Lee virus, as demonstrated by the lesser rise in titer in paired sera, but that the new strains may be antigenically broader in pattern, as shown by the ability of all second sera to inhibit hemagglutination by Lee significantly, whereas specific Lee serum did not inhibit hemagglutination by the new strains.

Strain differences in influenza B virus have been reported previously by Eaton and Beck,<sup>7</sup> Gordon,<sup>8</sup> Burnet *et al.*,<sup>9</sup> and others. However, it is considered of value to record the continued antigenic heterogeneity of this virus, particularly in view of the problem of choice of strains for inclusion in influenza vaccines.

*Summarizing*, there is reported an outbreak of influenza on the University of Washington campus, from which 3 strains of influenza virus B were isolated. Paired sera from 22 patients showed a significant rise in hemagglutination-inhibition titer against Lee virus in all cases and against the new strains in 18 sera. Because of the inability of rooster antiserum prepared against Lee virus to inhibit hemagglutination by the Seattle strains, it appears that these new viruses are incompletely related to the standard type B virus.

<sup>5</sup> Meiklejohn, G., personal communication, Feb. 11, 1949.

<sup>6</sup> Magill, T. P., personal communication, March 18, 1949.

<sup>7</sup> Eaton, M. D., and Beck, M. D., *Proc. Soc. Exp. Biol. and Med.*, 1941, 48, 177.

<sup>8</sup> Gordon, I., *J. Immun.*, 1942, 44, 231.

<sup>9</sup> Burnet, F. M., Beveridge, W. I. B., and Bull, D. R., *Aust. J. Exp. Biol. and Med. Sci.*, 1944, 22, 9.

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# 17178. Some Reactions of the Rat to Treatment with Progesterone and Estrone in Late Pregnancy.\*

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Following the observation that extract of corpora lutea<sup>1</sup> prolonged pregnancy in the rat, reports on the effects of estrogens<sup>2-4</sup> and progesterone<sup>4,5</sup> on parturition in the rat have appeared. These reports have not included observations on the behavior of the treated animals at the normal time for delivery. Because pregnancy was allowed to continue the viability of the young at the normal time for delivery was not determined. The purpose of the present study is to observe viability of the young and labor in rats treated daily during late pregnancy with estrone, progesterone or a combination of estrone and progesterone.

**Methods.** In the strain of rats (Anheuser-Busch) used in these experiments the young were normally delivered in the night following the 21st day of pregnancy or in the morning of the 22nd day. The day that sperms were observed in the vaginal smear was counted as the first day of pregnancy. The hormone injection was continued daily from the time of the first injection. The young were removed in the afternoon of the 22nd day by hysterectomy in animals that had not delivered spontaneously. The passage of blood from the vagina was regarded as evidence of uterine bleeding. Confirmatory evidence of uterine bleeding was obtained when the young were removed.

**Results.** A summary of the results ob-

tained is shown in Table I. Although uterine bleeding occurred in the 3 animals given 1 mg of progesterone, attempts to deliver the young were seen in only 1. The other 2 rats were quiescent and contraction of abdominal muscles observed prior to parturition in normal animals was not seen. At the time of removal 20% of the young were dead. No labor attitudes or uterine bleeding occurred in 4 animals given 2.5 mg of progesterone and these animals remained quiet and showed no evidence of efforts to deliver the young. Only 3% of the young were dead at the time of removal.

The well-known tendency of estrogen to terminate pregnancy led us to attempt to bring about spontaneous delivery of young in late pregnancy. The data in Table I indicate that administration of estrone in late pregnancy may either prevent parturition or accelerate delivery. It was found that daily injection of 25  $\gamma$  of estrone started on the 19th or 20th day of pregnancy caused prolonged labor but failure to deliver the young by the end of the 22nd day. One animal given 100  $\gamma$  of estrone on the 20th day delivered young 1 day premature less than 20 hours after the injection. In 4 animals treated with 25  $\gamma$  of estrone about 24 hours before time for delivery parturition seemed to be accelerated rather than prevented; about 12 to 18 hours after the first injection the animals became extremely active and delivered the young promptly. A point of particular interest is the sharp contrast in behavior of the estrone-treated animals as compared with the ones given progesterone. Those estrone-treated animals requiring removal of the young showed vigorous and prolonged efforts to deliver spontaneously. In the rats treated with estrone 40% of the young were dead on the 22nd day of pregnancy.

\* Aided by a grant from the U. S. Public Health Service.

<sup>1</sup> Nelson, W. O., Piffner, J. J., and Haterius, H. O., *Am. J. Physiol.*, 1930, **91**, 690.

<sup>2</sup> Hain, A. M., *Quart. J. Exp. Physiol.*, 1935, **25**, 131.

<sup>3</sup> D'Amour, F. E., and Dumont, C., *Quart. J. Exp. Physiol.*, 1937, **26**, 215.

<sup>4</sup> Boe, F., *Acta path. et Microbiol. Scandinav.*, suppl. 36, 1938.

<sup>5</sup> Imaz, F. A. U., *Prensa Méd. Argent.*, 1943, **30**, 2309.

TABLE I.  
Effects of Progesterone and Estrone in Late Pregnancy in the Rat.

Rat No.	Treatment daily*	Day begun	Delivery	Fetuses	Remarks
31	1 mg P†	19	Removed§	5 living, 4 dead	No labor. Uterine bleeding
32	1 mg P	19	"	11 "	" " " " "
35	1 mg P	19	"	4 " 1 "	Labor. Uterine bleeding
42	2.5 mg P	19	"	10 "	No labor. No uterine bleeding
44	2.5 mg P	19	"	9 "	" " " " "
45	2.5 mg P	19	"	7 "	" " " " "
46	2.5 mg P	19	"	9 "	" " " " "
50	200 γ E‡	19	Autopsy	8 "	Died 24th day after prolonged labor
51	25 γ E	19	Removed	2 " 5 "	Labor. Uterine bleeding
55	100 γ E	20	Spontaneous	11 "	1 day premature
56	25 γ E	20	Removed	1 " 6 "	Labor. Uterine bleeding
63	25 γ E	21	Spontaneous	3 " 3 "	22nd day of pregnancy
64	25 γ E	21	"	7 "	" " " " "
65	25 γ E	21	"	7 "	" " " " "
66	25 γ E	21	"	11 "	" " " " "
71	25 γ E	18	"	4 " 5 "	Parturition delayed 1 day. Prolonged labor
81	100 γ E + 1 mg P	19	Removed	7 " 2 "	No labor. Uterine bleeding
82	100 γ E + 1 mg P	20	"	8 "	" " " " "
92	25 γ E + 2.5 mg P	20	"	10 "	No labor. No uterine bleeding
93	25 γ E + 2.5 mg P	20	"	8 "	" " " " "
1-5	None	—	Spontaneous	43 " 2 "	22nd day of pregnancy

\* Except in rat No. 71, which received single injection.

† P = Progesterone.

‡ E = Estrone.

§ In all animals except Nos. 50 and 71, the young were delivered by hysterectomy toward end of 22nd day of pregnancy, if young were not delivered spontaneously.

The rats injected simultaneously with estrone and progesterone showed no effort to deliver the young and remained relatively inactive. It is possible, however, that labor may have occurred in some of the animals during the night when the animals were not under observation. Uterine bleeding was seen only in the 2 animals given large amounts of estrone. Only 6% of the young were dead.

**Discussion.** In only 2 studies of which we are aware has the daily injection of crystalline progesterone been continued in the rat beyond the normal term of pregnancy. Pregnancy was prolonged by daily injection of 1 mg of progesterone started the 18th day of pregnancy<sup>4</sup> and by 0.5 mg daily started on the 16th day.<sup>5</sup> The observation<sup>6</sup> that 2 Corner-Allen Rb. U. (2 mg) of progesterone are required to maintain pregnancy in the ovariectomized rat suggested to us that the dosages of this hormone used in the above

studies on parturition may not completely prevent labor. In the rabbit, for example, it has been shown that 1 mg of progesterone maintains pregnancy after removal of the ovaries<sup>7</sup> while 1.5 mg is required to prolong pregnancy significantly.<sup>8</sup> Our results show that 2.5 mg of progesterone daily are required to prevent uterine bleeding when injections are begun on the 19th day of pregnancy.

It has been shown<sup>4</sup> that 600 γ and 300 γ of estrone benzoate given daily from the 16th to the 22nd day of pregnancy in the rat kills most of the young and delays delivery. Similar results<sup>2</sup> were obtained in the rat with daily injection of 125 γ to 500 γ of estrogens begun late in pregnancy. Heckel and Allen<sup>9</sup>

<sup>7</sup> Allen, W. M., and Corner, G. W., *Proc. Soc. Exp. Biol. and Med.*, 1930, **27**, 493.

<sup>8</sup> Heckel, G. P., and Allen, W. M., *Am. J. Physiol.*, 1937, **119**, 330.

<sup>9</sup> Heckel, G. P., and Allen, W. M., *Endocrinology*, 1939, **24**, 137.

<sup>6</sup> Rothschild, L., and Meyer, R. K., *Proc. Soc. Exp. Biol. and Med.*, 1940, **44**, 402.

# 17178. Some Reactions of the Rat to Treatment with Progesterone and Estrone in Late Pregnancy.\*

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Following the observation that extract of corpora lutea<sup>1</sup> prolonged pregnancy in the rat, reports on the effects of estrogens<sup>2-4</sup> and progesterone<sup>4,5</sup> on parturition in the rat have appeared. These reports have not included observations on the behavior of the treated animals at the normal time for delivery. Because pregnancy was allowed to continue the viability of the young at the normal time for delivery was not determined. The purpose of the present study is to observe viability of the young and labor in rats treated daily during late pregnancy with estrone, progesterone or a combination of estrone and progesterone.

**Methods.** In the strain of rats (Anheuser-Busch) used in these experiments the young were normally delivered in the night following the 21st day of pregnancy or in the morning of the 22nd day. The day that sperms were observed in the vaginal smear was counted as the first day of pregnancy. The hormone injection was continued daily from the time of the first injection. The young were removed in the afternoon of the 22nd day by hysterectomy in animals that had not delivered spontaneously. The passage of blood from the vagina was regarded as evidence of uterine bleeding. Confirmatory evidence of uterine bleeding was obtained when the young were removed.

**Results.** A summary of the results ob-

tained is shown in Table I. Although uterine bleeding occurred in the 3 animals given 1 mg of progesterone, attempts to deliver the young were seen in only 1. The other 2 rats were quiescent and contraction of abdominal muscles observed prior to parturition in normal animals was not seen. At the time of removal 20% of the young were dead. No labor attitudes or uterine bleeding occurred in 4 animals given 2.5 mg of progesterone and these animals remained quiet and showed no evidence of efforts to deliver the young. Only 3% of the young were dead at the time of removal.

The well-known tendency of estrogen to terminate pregnancy led us to attempt to bring about spontaneous delivery of young in late pregnancy. The data in Table I indicate that administration of estrone in late pregnancy may either prevent parturition or accelerate delivery. It was found that daily injection of 25  $\gamma$  of estrone started on the 19th or 20th day of pregnancy caused prolonged labor but failure to deliver the young by the end of the 22nd day. One animal given 100  $\gamma$  of estrone on the 20th day delivered young 1 day premature less than 20 hours after the injection. In 4 animals treated with 25  $\gamma$  of estrone about 24 hours before time for delivery parturition seemed to be accelerated rather than prevented; about 12 to 18 hours after the first injection the animals became extremely active and delivered the young promptly. A point of particular interest is the sharp contrast in behavior of the estrone-treated animals as compared with the ones given progesterone. Those estrone-treated animals requiring removal of the young showed vigorous and prolonged efforts to deliver spontaneously. In the rats treated with estrone 40% of the young were dead on the 22nd day of pregnancy.

\* Aided by a grant from the U. S. Public Health Service.

<sup>1</sup> Nelson, W. O., Pflüger, J. J., and Haterius, H. O., *Am. J. Physiol.*, 1930, **91**, 690.

<sup>2</sup> Hain, A. M., *Quart. J. Exp. Physiol.*, 1935, **25**, 131.

<sup>3</sup> D'Amour, F. E., and Dumont, C., *Quart. J. Exp. Physiol.*, 1937, **26**, 215.

<sup>4</sup> Boe, F., *Acta path. et Microbiol. Scandinav.*, suppl. 36, 1938.

<sup>5</sup> Imaz, F. A. U., *Prensa Méd. Argent.*, 1943, **30**, 2309.

TABLE I.  
 Effect of NaPAH on Urinary Phosphate Excretion.

Period No. and specimen		Concurrent time, min.	Urine volume, cc/min./ 1.73 M <sup>2</sup>	PAH conc., mg %	Phosphate conc., m.Osm/L	Phosphate excreted, m.Osm/min./ 1.73 M <sup>2</sup>
L.J. Hydration.	9 yrs.	S.A. 1.25 M <sup>2</sup> .	Wt. 37.3 kg.			
P-1	U	-66 to -35	1.10		12.3	.014
P-2	U	-35 to -10	8.39		2.0	.017
	P	-1			1.50	
		0 to 10 I.V. injection 50 cc 20% NaPAH.				
1	U	24 to 38	3.31	3800	11.0	.036
	P	28		62.2	1.54	
2	U	58 to 75	4.61	1529	6.8	.031
	P	69		14.1	1.56	
3	U	75 to 97	6.13	739	4.4	.027
4	U	97 to 118	8.00	254	3.0	.024
W.H. Dehydration.	11 yrs.	S.A. 1.12 M <sup>2</sup> .	Wt. 30.9 kg.			
P-1	U	-116 to -86	0.38		53.2	.020
P-2	U	-86 to -59	0.31		59.4	.018
P-3	U	-59 to -23	0.28		67.8	.019
	P	-1			1.61	
		0 to 5 I.V. injection 35 cc 20% NaPAH.				
1	U	22 to 42	2.88	4560	19.4	.056
	P	25		54.3	1.54	
2	U	42 to 63	1.63	4622	28.7	.047
	P	56		13.6	1.52	
3	U	63 to 82	0.82	4114	46.4	.038

affect the renal excretion of several substances;<sup>2-4</sup> an effect on phosphate excretion has not been previously described. This report concerns studies of phosphate excretion at various plasma PAH levels under conditions of both hydration and dehydration.

**Procedure and methods.** The studies were done on one female and 5 male patients, 9 to 12 years of age, who showed no evidence of renal disease. One child had slight albuminuria and hematuria associated with an episode of acute rheumatic fever three weeks previous to the experiment but subsequently has shown no evidence of renal disease. The subjects were in the post-absorptive state. Two subjects were deprived of water for 16 hours prior to the procedure. In these the PAH produced osmotic diuresis. In the other patients, high urine flows were maintained by water intake as is commonly done for renal clearance studies.

The general procedure of 4 experiments was

as follows: After the collection of several preliminary urine specimens and one blood specimen, sufficient 20% NaPAH was injected intravenously in a 10 to 15 minute interval to produce plasma levels of 60 mg % or higher. Four to six urine specimens at 15 to 20 minute intervals and 2 or more blood specimens were then collected.

In 2 of the hydrated subjects, the procedure was somewhat modified as will be described later.

In blood and urine, PAH was determined by the method of Smith *et al.*<sup>5</sup> and phosphate by the method of Fiske and Subbarow.<sup>6</sup> Urine volumes were corrected to 1.73 M<sup>2</sup> for the calculation of minute phosphate excretion.

**Results.** The pertinent data of 2 representative experiments during hydration and dehydration are given in Table I. For all experiments, phosphate excretion during preliminary urine periods prior to the injection of NaPAH ranged from 0.002 to 0.020 mOsmols min. In every case the rate of ex-

<sup>5</sup> Smith, H. W., Finkelstein, N., Aliminosa, L., Crawford, B., and Graber, M., *J. Clin. Invest.*, 1945, **24**, 388.

<sup>6</sup> Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, 1925, **66**, 375.

<sup>2</sup> Selkurt, E. E., *Am. J. Physiol.*, 1944, **142**, 192.

<sup>3</sup> Beyer, K. H., Woodward, R., Peters, L., Verwey, W. F., and Mattis, P. A., *Science*, 1944, **100**, 107.

<sup>4</sup> Houck, C. R., *Proc. Soc. Exp. Biol. and Med.*, 1946, **61**, 398.



found that during daily injection of estrone in pregnant rabbits beginning on the 29th day 400 I.U. killed the young but did not significantly delay parturition and 1000 I.U. prevented delivery; delivery occurred normally on the 32nd day in this strain of rabbits. The prevention of parturition in the rabbit was regarded as due primarily to prolongation of the functional state of corpora lutea. The prolongation of pregnancy in the hypophysectomized rabbit<sup>10</sup> and in the rat<sup>11</sup> by treatment with estrogens was similarly interpreted. Our findings that estrone injection started on the 19th day of pregnancy in the rat brought about vigorous but ineffective efforts to deliver young while progesterone treatment started on the same day induced quiescence, suggest that estrone plays a role in the prevention of parturition in the rat other than by way of its action on the corpora lutea. Because estrone apparently increases the frequency and intensity of contraction of abdominal muscle, it is suggested that failure to deliver the young is due to obstruction of the birth canal possibly as a result of failure of relaxation in the region of the cervix. In the rat wide abdominal incision during parturition practically stops transport and expulsion of the fetuses and closure of the incision restores both processes.<sup>3</sup> This observation, which was interpreted as evidence that contractions of the diaphragm and ab-

dominal muscles provide the primary force for delivery of young, supports our view that failure of expulsion in estrone-treated rats showing vigorous labor is due to failure of uterine relaxation.

Our observation that progesterone injection started simultaneously with estrone treatment on the 19th or 20th day of pregnancy prevents labor, uterine bleeding and death of young suggests that the death of fetuses induced by estrone may be due, at least in part, to intense but ineffective labor.

*Summary.* Progesterone (2.5 mg daily) beginning on the 19th day of pregnancy in intact rats prevents labor, uterine bleeding and parturition. Estrone (25  $\gamma$ ) started on the 19th or 20th day induces uterine bleeding, vigorous but ineffective labor and death of fetuses. Estrone (100  $\gamma$ ) begun on the 20th day in 1 animal brought about delivery of young 1 day premature. Spontaneous delivery occurred in 4 rats given 25  $\gamma$  of estrone about 24 hours before time for delivery. Progesterone (2.5 mg) given simultaneously with estrone (25  $\gamma$ ) beginning on the 20th day has the same effect as progesterone alone, preventing the above effects of estrone alone. It is suggested that estrone plays a role in the prevention of parturition in the rat other than by way of its action on the corpora lutea.

Progesterone from Schering Corporation, Bloomfield, N. J., and estrone from Parke Davis and Co., Detroit, Mich., were generously supplied for this study.

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<sup>10</sup> Robson, J. M., *J. Physiol.*, 1940, 97, 517.

<sup>11</sup> Odendaal, W. A., *South African J. M. Sc.*, 1944, 9, 131.

## 17179. Urinary Excretion of Phosphate Following the Injection of Sodium p-Aminohippurate.

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In the course of recent studies of osmotic diuresis in man,<sup>1</sup> the rate of urinary excretion

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of phosphate was found to be increased following the injection of sodium p-aminohippurate. Although PAH has been found to

<sup>1</sup> Rapoport, S., West, C. D., and Brodsky, W. A., *Am. J. Physiol.*, in press.

tion by PAH is thus of considerable magnitude but is not sufficient to cause excretion to occur at the level of glomerular filtration.

It is worthy of note that the diminished tubular reabsorption of phosphate which occurs after injection of parathyroid hormone is of about the same order of magnitude. The data of Harrison and Harrison<sup>7</sup> on dogs show that after injection of parathyroid extract, 25 to 28% of the filtered phosphate was excreted. Filtration rates were determined by creatinine clearance in these experiments. In normal adults the ratio of excreted to filtered phosphate after parathyroid hormone injection can be calculated from the data of other workers, assuming a filtration rate of 120 cc./min. The 5 subjects of Ellsworth and Howard<sup>8</sup> excreted 11 to 41% of the phosphate filtered in the second and third hours after injection of 40 units of parathyroid hormone. Essentially the same percentage of phosphate excreted may be calculated from the data for the two subjects of Goadby and Stacey<sup>9</sup> after doses of 60 and 100 units of parathyroid extract.

Previous work has shown that the tubular transport of a number of substances may be depressed by PAH. The tubular reabsorption of ascorbic acid<sup>2</sup> and the tubular excretion of penicillin<sup>3</sup> are reduced by the presence of high plasma PAH levels. The inhibition of tubular transport of these substances may be so great that excretion occurs at the level of glomerular filtration. Glucose and PAH appear to inhibit reciprocally the tubular transfer of each other. In dogs the  $T_m$  for glucose has been shown to be consistently depressed by high PAH levels.<sup>4</sup> In man, the opposite effect, an increased  $T_m$  for glucose in the presence of high PAH levels, has been reported by Klopp, Young, and Taylor<sup>10</sup> in 2 out of 4 patients studied. An explanation of this unexpected finding may lie in the fact that in their experiments, the mannitol clearance, used as a measure of

glomerular filtration rate, was found to be increased significantly with high PAH levels. It is possible that the augmentation of the mannitol clearance and consequently of the glucose  $T_m$  was the result of an error in the determination of mannitol due to interference by PAH. Barker and Clark<sup>11</sup> have recently shown that in the method used by Klopp *et al.* (periodate-iodide-thiosulfate titrimetric procedure), PAH causes a large positive error, to the extent of 0.27 mg of mannitol per mg of PAH. The reciprocal effect, *i.e.*, inhibition of the transport of PAH by high plasma glucose levels has been consistently demonstrated in both dog and man.<sup>4,10,12,13</sup>

The nature of the inhibition of tubular transport can only be speculated upon. The inhibition of creatine reabsorption by glycine, alanine and glutamic acid<sup>14,15</sup> and of xylose reabsorption by glucose<sup>16</sup> has been attributed to competition for a common mechanism of limited capacity involving a single transport substance (B substance of Shannon<sup>17</sup>). Although such an explanation may be valid for structurally similar substances, it is difficult to conceive of one enzymatic or chemical mechanism sufficiently versatile to act in such a widely differing manner as to secrete PAH and penicillin or reabsorb ascorbic acid, glucose, and the phosphate ion. The circumstance that 4 of the 5 substances just mentioned are anions would suggest the existence of a tubular transport mechanism based on this property. However, the mutual inhibition of glucose and PAH speaks strongly against such an assumption. Another point, possibly contradicting the idea of a common mechanism, is the fact that one of the sub-

<sup>10</sup> Klopp, C., Young, N. F., and Taylor, H. C., Jr., *J. Clin. Invest.*, 1945, **24**, 117.

<sup>11</sup> Barker, H. G., and Clark, J. K., *Proc. Soc. Exp. Biol. and Med.*, 1947, **64**, 120.

<sup>12</sup> Grimelli, L. J., Chertack, M. M., Rhetta, H. L., Kendrick, A. B., and Forrest, R. A., *J. Lab. and Clin. Med.*, 1948, **33**, 1617.

<sup>13</sup> Kelley, V. C., and McDonald, R. K., *Am. J. Physiol.*, 1948, **154**, 201.

<sup>14</sup> Pitts, R. F., *Am. J. Physiol.*, 1943, **140**, 156.

<sup>15</sup> Pitts, R. F., *Am. J. Physiol.*, 1944, **140**, 535.

<sup>16</sup> Shannon, J. A., *Am. J. Physiol.*, 1938, **122**, 775.

<sup>17</sup> Shannon, J. A., *Physiol. Revs.*, 1939, **19**, 63.

<sup>7</sup> Harrison, H. E., and Harrison, H. C., *J. Clin. Invest.*, 1941, **20**, 47.

<sup>8</sup> Ellsworth, R., and Howard, J., *Bull. Johns Hopkins Hosp.*, 1934, **55**, 296.

<sup>9</sup> Goadby, H. K., and Stacey, R. S., *Biochem. J.*, 1934, **28**, 2092.

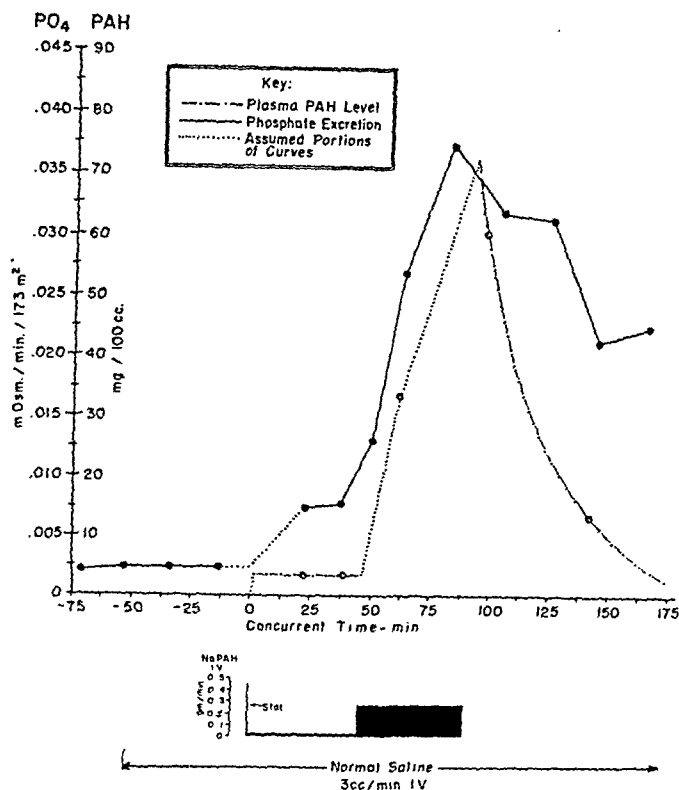


FIG. 1.

Phosphate excretion and plasma PAH level. See text for details.

cretion increased following the injection of NaPAH, regardless of the state of hydration of the subject. The maximum rate usually occurred during the first or second collection period following the rapid injection, not always coincident with the highest plasma PAH concentration. The maximum rate of phosphaturia ranged from 0.017 to 0.069 mOsmols/min., representing 2.3 to 16 fold increases over the respective rates during preliminary periods. After reaching a maximum, the excretion diminished at a rate coinciding roughly with the fall in plasma PAH concentration.

Plasma phosphate concentration did not change significantly during the course of the experiments. The phosphate level averaged 1.40 mOsmols/liter for six specimens taken prior to the injection of NaPAH and 1.46 mOsmols/liter for 19 specimens taken after the injection.

In Fig. 1 is presented one of two experiments so arranged as to observe phosphate

excretion at constant low plasma PAH levels (3 to 5 mg %) and during the rise to and fall from higher concentrations. Throughout the experiment, with the exception of the first two preliminary periods, normal saline was injected intravenously at a constant rate of 3.0 cc/min. with sufficient NaPAH added at intervals to produce the plasma concentrations shown. The effect of PAH in increasing the rate of phosphate excretion is readily apparent.

*Discussion.* Under normal conditions extensive tubular reabsorption of phosphate takes place. Assuming a filtration rate of 120 cc/min., the amount of phosphate excreted during the preliminary periods of the experiments here presented averaged 7% of the amount filtered. After injection of NaPAH during the periods of greatest phosphaturia, assuming the same filtration rate, the phosphate excreted ranged from 9 to 45%, with an average of 26% of the amount filtered. The inhibition of tubular phosphate reabsorp-

TABLE I.  
Effects of Dietary Thyroid and Cholesterol in Male Rats.  
Figures are Average Oxygen Consumption and Standard Error.

Group <sup>a</sup>	Thyroid fed	Cholesterol fed	At onset	After 3 wks	After 6 wks	After 9 wks
			ml oxygen/100 g body wt/lir			
N	—	—	123 ± 4	106 ± 3	108 ± 4	—
C	—	+	121 ± 2	106 ± 3	105 ± 3	—
T	+	—	122 ± 2	166 ± 4	162 ± 6	160 ± 6
T + C	+	+	121 ± 4	157 ± 3	144 ± 3	144 ± 4

\* Each group consisted of 8 rats.

TABLE II.  
Effects of Dietary Thyroid and Cholesterol in Male Rats. Average Body Weight Gain, Food Intake, and Tissue Cholesterol Content.

Group <sup>a</sup>	Thyroid fed	Cholesterol fed	Body wt gain in 9 wks and standard error, g	Food intake,† g/100 g body wt/day	Cholesterol content†	
					Plasma, mg/100 ml	Liver, %
N	—	—	122 ± 5	5.7	53	0.21
C	—	+	109 ± 7	5.9	62	0.79
T	+	—	80 ± 7	7.1	49	0.24
T + C	+	+	72 ± 8	8.2	54	0.81

\* Each group consisted of 8 rats.

† No standard errors are given, since, in each group, the samples were pooled in lots of 2 or 3.

of the experimental diets was begun, and the oxygen consumption was determined every 3 weeks. Body weights were recorded every 10 days, and the food consumption was measured at 2 day intervals during the seventh and eighth week.

After an experimental period of 9 weeks, the animals were autopsied, and weights and total cholesterol contents of various tissues were measured, the latter according to a modified Schoenheimer-Sperry-Chaney procedure.<sup>5</sup>

**Results.** The data on the determination of the oxygen consumption indicate that the administration of thyroid raised the metabolic rate by about 60% (Table I). The addition of cholesterol to the high thyroid diet caused a reduction of this value by about 10%, the difference being statistically significant ( $P < 0.01$  for the 6 week period;  $P =$  approximately 0.025 for the 9 week period). The metabolic rate of normal rats was not significantly modified by the addition of cholesterol to the diet.

Thyroid administration depressed the body weight gain and stimulated the animal's appetite, as was to be expected (Table II). The

addition of cholesterol to the diet did not modify the food intake significantly, neither in normal nor in hyperthyroid rats. It is obvious, therefore, that the depressing effect of dietary cholesterol on the metabolic rate can not be attributed to a decrease in food intake.

Liver weights were found increased as a consequence of both thyroid and cholesterol administration, group T + C showing the highest values. Thyroid hormone, but not cholesterol, caused significant enlargement of the kidneys and adrenals also. These changes were to be expected.

Although total cholesterol concentrations of the plasma were not much modified, the level of liver cholesterol was markedly increased by cholesterol feeding, regardless of the thyroid content of the diet (Table II).

**Discussion.** Although cholesterol feeding significantly reduced the oxygen consumption of the hyperthyroid rats, the decrease amounted to only about 10% and was not sufficient for the metabolic rate to approach normal levels. The recently reported protective action of cholesterol as regards the survival time of thyrotoxic rats is much more pronounced;<sup>1,2</sup> it is questionable, therefore, whether this effect on the survival time is based on a mechanism related to the meta-

<sup>4</sup> Mason, G. D., and Winzler, R. J., to be published. The authors are much obliged to Dr. Winzler for the privilege of using this apparatus.

<sup>5</sup> To be published.

stances, phosphate, is an inorganic ion.

The inhibition of tubular transport by PAH may be more adequately explained by the hypothesis of a common energy source of limited capacity. Competition for the available energy by the activity of the mechanism for the transport of one solute would then result in diminished transport of another solute. Neither the locus nor the character of the energy limitation can be stated. Some of the possible mechanisms involved have been discussed by Selkurt<sup>2</sup> and by Houck.<sup>4</sup>

It should be emphasized that the inhibition

of tubular transport of one substance by another may result in serious error when several renal functions are measured simultaneously.

**Summary.** The urinary excretion of phosphate is increased by the injection of sodium p-aminohippurate in man. The extent of the phosphaturia tends to vary directly with the plasma PAH level. At high PAH levels, the amount of phosphate excreted averages 26% of that filtered, a proportion comparable in magnitude to that reported after injection of parathyroid hormone.

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### 17180. Effect of Dietary Cholesterol on the Metabolic Rate of Hyperthyroid Rats.\*

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It was recently observed that dietary cholesterol increases the survival time of rats fed toxic doses of thyroid hormone.<sup>1,2</sup> In an attempt to learn whether this protective action of cholesterol is correlated with a corresponding modification of the effects of thyroid on the metabolic rate, the influence of dietary cholesterol was investigated on the oxygen consumption of hyperthyroid rats.

**Methods.** Thirty-two male rats (USC strain), about 9 weeks of age, were distributed evenly among 4 groups which received the following diets *ad libitum*: I. This laboratory's stock diet, slightly modified to con-

tain more vitamin B complex<sup>‡</sup> ("N"). II. Diet I, containing, in addition, 1 to 2% cholesterol<sup>§</sup> ("C"). III. Diet I, containing, in addition, 0.15% desiccated thyroid U.S.P.<sup>§</sup> ("T"). IV. Diet I, containing, in addition, both 0.15% thyroid and 1 to 2% cholesterol. ("T + C"). At first, cholesterol was fed at a level of 1%, but after 3 weeks, its concentration in the diet was raised to 2%. The cholesterol was dissolved in hot cottonseed oil, replacing a corresponding amount of oil in the diet. In order to increase cholesterol absorption, 0.25% bile salt<sup>§</sup> was added to all diets.<sup>3</sup>

The oxygen consumption was determined using a closed circuit apparatus provided with separate chambers for individual animals.<sup>4</sup> After repeated measurements during a preliminary training period of 5 weeks, feeding

\* These data are from a thesis presented by Louis E. Winebrenner to the Graduate School of the University of Southern California in partial fulfillment of the degree of Master of Science. The authors wish to express their appreciation for the use of the facilities of the Hancock Foundation. Paper No. 219 from the Department of Biochemistry and Nutrition.

<sup>†</sup> Captain, USAF. Present address: Hq. USAF, Director of Armament, Pentagon Building, Washington, D.C.

<sup>1</sup> Marx, W., Meserve, E. R., and Deuel, H. J., Jr., *Proc. Soc. Exp. Biol. and Med.*, 1948, **67**, 385.

<sup>2</sup> Ershoff, B. H., and Marx, W., *Exp. Med. and Surg.*, 1948, **6**, 145.

<sup>‡</sup> Whole wheat, 32.5%; oats, 32.75%; skimmed milk, 10%; alfalfa meal, 4%; yeast, Anheuser-Busch strain G, 9.5%; Wesson oil, 8%; fortified oil containing 1500 I.U. vitamin A/g and 160 I.U. vitamin D<sub>2</sub>/g, 2%; sodium chloride, 0.5%; calcium carbonate, 0.5%; bile salt, 0.25%.

<sup>§</sup> Cholesterin c.p., Amend Drug and Chemical Co., New York City; desiccated thyroid, U.S.P., Armour and Co., Chicago, Ill.; sodium glycocholate (pure), City Chemical Corp., New York City.

<sup>3</sup> Schoenheimer, R., *Biochem. Z.*, 1924, **147**, 258.

slanting position at 34°C. Supernatant fluids were replaced each 7 to 14 days, *i.e.*, as the pH fell from 7.5 to 7.0. The plasma was patched at these intervals as required. All cultures were examined each week and, until their shape became irregular as they almost filled the plasma, measured along two diameters by means of an ocular micrometer. For the present purpose additional notes were made on the pH and the turbidity of the media, the occurrence of precipitate in the liquid phase, and on the opacity of the explants and of the plasma.

The chicken plasma (*CH*) contained 0.2% sodium citrate. Chick embryos were pressed through a Latapie grinder and extracted with 2 ml balanced salt solution (lacking  $\text{NaHCO}_3$ ) per gram of embryo. These suspensions were spun immediately in the SP angle centrifuge for 30 minutes at 3000 RPM. The supernatants, after having been respun in the same way, were remarkably clear except for an upper stratum of lipid. When adjusted to neutrality and buffered with 0.125%  $\text{NaHCO}_3$ , the concentration of these extracts became 30% (*EM*<sub>30</sub>). The salt solution, Sol. A, (otherwise as before)<sup>5</sup> contained 0.2 g per liter of  $\text{CaCl}_2$  and 0.06 g each of  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$  and of  $\text{KH}_2\text{PO}_4$ . After adding to the autoclaved salt solution 10% by volume of sterile 1.4%  $\text{NaHCO}_3$ , the concentration of bicarbonate became 0.127%, of Ca 6.5 mg%, and of inorganic P 2.2 mg%. A similar Sol. N (no Ca or P) and other modifications will be mentioned in the text.

In the early work placental serum (PLS) was collected as available and preserved by Chrochem dehydration. When reconstituted, 0.1% glucose and 0.002% phenol red were included and the serum further diluted to 50% in the balanced salt solution. It was sterilized by pressure filtration (5%  $\text{CO}_2$ ) through a Seitz filter. The Seitz pads were found to contribute Mg ions and alkaline radicals, and this method was replaced by aseptic collection of serum. The composition of fluid media will be indicated in code, *e.g.*, *PLS*<sub>25</sub>*EM*<sub>15</sub>, referring to placental serum 25%, embryo juice 15%.

**Chemical determination.** Both calcium and inorganic phosphate were determined in

trichloroacetic acid filtrates. Calcium was determined by Tisdall's modification<sup>6</sup> of Kramer and Tisdall's method and phosphorus by Brigg's<sup>7</sup> modification of the Bell and Doisy method. Values for Ca and inorganic P in mg% will be written as exponents.  $\text{Ca} \times \text{P}$  will designate the product of these values. For example,  $\text{Ca} \times \text{P}$  of the salt solution:  $\text{Ca}^{6.5} \times \text{P}^{2.2} = 14.3$ .

**Experimental results.** After many early attempts to employ 15% chick embryo juice in the presence of 25% human serum, it became evident that several conditions influenced the calcification. These factors included: the age of the embryos employed, the proportion of embryo juice mixed with the serum, and different methods of preparing, storing, or treating the embryo juice.

**Age of Embryos.** Various batches of extract from pools of 10-day-incubated embryos differed remarkably in their ability to cause calcification. Since Philippine chickens hide their nests, overage eggs were unavoidable among the freshest obtainable. On one occasion marked calcification resulted from a single application of medium containing embryo juice from a pool of 5 embryos, one of which was perhaps 12 or 13 days actual age. From such observations it became apparent that calcification *in vitro* was related to the development of the calcifying mechanisms of the chick embryo. Data eventually collected on Ca and P values in relation to the age and weight of embryos (see Table I) revealed that the  $\text{Ca} \times \text{P}$  of the embryo juice averaged 68.5 by the 11th day of incubation and reached approximately 100 by the 12th day. It will be noted that the typical Ca : P ratios at 11 days average 1:2.4 and are *inverted by comparison with mammalian ratios*.

Comparisons between extracts from 9- and 11-day embryos from the same lots of eggs revealed that the juice from the 11-day embryos (in *PLS*<sub>25</sub>*EM*<sub>15</sub>) usually induced calcification after second renewal of the medium while juice from the 9-day embryos permitted 3 or 4 renewals. 9-day extracts, however, were inferior to 11-day juices for growth stimulation and for continuous main-

<sup>6</sup> Tisdall, F. F., *J. Biol. Chem.*, 1923, 56, 439.

<sup>7</sup> Briggs, A. P., *J. Biol. Chem.*, 1922, 53, 13.

bolic rate.

The results reported here may be related, however, to observations of Saegesser,<sup>6</sup> who reported a depression by cholesterol of thyroxine effects on the rate of metamorphosis of tadpoles, and on the oxygen requirement of rats in the oxygen deficiency test ("Sauerstoffmangelversuch") of Asher and Streuli.<sup>7</sup>

<sup>6</sup> Saegesser, M., *Klin. Wochenschr.*, 1933, **12**, 672.

*Summary.* Dietary cholesterol significantly decreased the oxygen consumption of thyroid-fed rats, but the reduction was not sufficient for the metabolic rate to approach normal levels. This effect of cholesterol cannot be attributed to a depression of the food intake.

<sup>7</sup> Asher, L., and Streuli, H., *Biochem. Z.*, 1918, **87**, 359.

Received May 24, 1949. P.S.E.B.M., 1949, **71**.

## 17181. Calcification of Cell Cultures in the Presence of Embryo Juice and Mammalian Sera.

JOHN H. HANKS.

*From Leonard Wood Memorial, Department of Bacteriology and Immunology,\* Harvard Medical School, Boston, Mass.*

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Since chick embryos were the only source of tissue extract available at Culion, the factors causing calcification were studied and means developed for avoiding or minimizing these difficulties in the presence of mammalian sera.

*Methods.* Cultures were prepared as previously described,<sup>5</sup> except that 2% embryo juice was used to transfer the 1.5 mm explants of human skin, leproma, or divided tissue culture to the 13 mm tubes containing one drop of chicken plasma 50%. After adding 0.5 ml of liquid phase and equilibration under 30 mm (4%) CO<sub>2</sub>, the tubes were incubated in a

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<sup>5</sup> Hanks, J. H., *J. Cell. and Comp. Physiol.*, 1948, **31**, 235.

\* The greater part of the work was done in the Leonard Wood Memorial Laboratory, Culion, Philippines, 1941-43.

<sup>1</sup> Earle, W. R., *Am. J. Cancer*, 1931, **24**, 566.

slanting position at 34°C. Supernatant fluids were replaced each 7 to 14 days, *i.e.*, as the pH fell from 7.5 to 7.0. The plasma was patched at these intervals as required. All cultures were examined each week and, until their shape became irregular as they almost filled the plasma, measured along two diameters by means of an ocular micrometer. For the present purpose additional notes were made on the pH and the turbidity of the media, the occurrence of precipitate in the liquid phase, and on the opacity of the explants and of the plasma.

The chicken plasma (CH) contained 0.2% sodium citrate. Chick embryos were pressed through a Latapie grinder and extracted with 2 ml balanced salt solution (lacking  $\text{NaHCO}_3$ ) per gram of embryo. These suspensions were spun immediately in the SP angle centrifuge for 30 minutes at 3000 RPM. The supernatants, after having been respun in the same way, were remarkably clear except for an upper stratum of lipoid. When adjusted to neutrality and buffered with 0.125%  $\text{NaHCO}_3$ , the concentration of these extracts became 30% ( $EM_{30}$ ). The salt solution, Sol. A, (otherwise as before)<sup>5</sup> contained 0.2 g per liter of  $\text{CaCl}_2$  and 0.06 g each of  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$  and of  $\text{KH}_2\text{PO}_4$ . After adding to the autoclaved salt solution 10% by volume of sterile 1.4%  $\text{NaHCO}_3$ , the concentration of bicarbonate became 0.127%, of Ca 6.5 mg%, and of inorganic P 2.2 mg%. A similar Sol. N (no Ca or P) and other modifications will be mentioned in the text.

In the early work placental serum (PLS) was collected as available and preserved by Chrocochem dehydration. When reconstituted, 0.1% glucose and 0.002% phenol red were included and the serum further diluted to 50% in the balanced salt solution. It was sterilized by pressure filtration (5%  $\text{CO}_2$ ) through a Seitz filter. The Seitz pads were found to contribute Mg ions and alkaline radicals, and this method was replaced by aseptic collection of serum. The composition of fluid media will be indicated in code, *e.g.*,  $PLS_{25}EM_{15}$  referring to placental serum 25%, embryo juice 15%.

**Chemical determination.** Both calcium and inorganic phosphate were determined in

trichloroacetic acid filtrates. Calcium was determined by Tisdall's modification<sup>6</sup> of Kramer and Tisdall's method and phosphorus by Brigg's<sup>7</sup> modification of the Bell and Doisy method. Values for Ca and inorganic P in mg% will be written as exponents.  $\text{Ca} \times \text{P}$  will designate the product of these values. For example,  $\text{Ca} \times \text{P}$  of the salt solution:  $\text{Ca}^{6.5} \times \text{P}^{2.2} = 14.3$ .

**Experimental results.** After many early attempts to employ 15% chick embryo juice in the presence of 25% human serum, it became evident that several conditions influenced the calcification. These factors included: the age of the embryos employed, the proportion of embryo juice mixed with the serum, and different methods of preparing, storing, or treating the embryo juice.

**Age of Embryos.** Various batches of extract from pools of 10-day-incubated embryos differed remarkably in their ability to cause calcification. Since Philippine chickens hide their nests, overage eggs were unavoidable among the freshest obtainable. On one occasion marked calcification resulted from a single application of medium containing embryo juice from a pool of 5 embryos, one of which was perhaps 12 or 13 days actual age. From such observations it became apparent that calcification *in vitro* was related to the development of the calcifying mechanisms of the chick embryo. Data eventually collected on Ca and P values in relation to the age and weight of embryos (see Table I) revealed that the  $\text{Ca} \times \text{P}$  of the embryo juice averaged 68.5 by the 11th day of incubation and reached approximately 100 by the 12th day. It will be noted that the typical Ca : P ratios at 11 days average 1:2.4 and are *inverted by comparison with mammalian ratios*.

Comparisons between extracts from 9- and 11-day embryos from the same lots of eggs revealed that the juice from the 11-day embryos (in  $PLS_{25}EM_{15}$ ) usually induced calcification after second renewal of the medium while juice from the 9-day embryos permitted 3 or 4 renewals. 9-day extracts, however, were inferior to 11-day juices for growth stimulation and for continuous main-

<sup>6</sup> Tisdall, F. F., *J. Biol. Chem.*, 1923, 56, 439.

<sup>7</sup> Briggs, A. P., *J. Biol. Chem.*, 1922, 53, 13.



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*Summary.* Dietary cholesterol significantly decreased the oxygen consumption of thyroid-fed rats, but the reduction was not sufficient for the metabolic rate to approach normal levels. This effect of cholesterol cannot be attributed to a depression of the food intake.

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TABLE II.  
Ca and P Values Upon Mixing Various Proportions of Placental Serum 50% with 11-day Chick Embryo Juice 30%, and Their Relation to Calcification of Tissue Cultures.

Formula of medium %	Proportion of each component	Ca and P contributed by each		C : P ratio	Initial Ca x P product of medium	Applications required to produce calcification	Potential Ca x P product of medium
		Ca mg %	P mg %				
PLS 50*	6/6	9.0	5.0	1: 0.6	(45.0)		
EM 30*	6/6	6.0	13.0	1: 2.2	(78.0)		
PLS 17	2/6	3.0	1.7				
EM 20	4/6	4.0	8.7	1: 1.5	72.8	2	79.3
		T 7.0	10.4				
PLS 25	3/6	4.5	2.5				
EM 15	3/6	3.0	6.5	1: 1.2	67.5	3	74.8
		T 7.5	9.0				
PLS 33	4/6	6.0	3.3				
EM 10	2/6	2.0	4.3	1: 1.0	60.8	4 or 5	67.5
		T 8.0	7.6				
PLS 42	5/6	7.5	4.2				
EM 5	1/6	1.0	2.2	1: 0.8	54.4	6 to 8	58.1
		T 8.5	6.4				

\* Composition of ingredients used to prepare the medium.

Method of calculating initial Ca : P and Ca x P shown in detail.

T = Total Ca and P upon making mixtures. Potential Ca x P calculated in similar manner, using the Ca and P values of EM<sub>30</sub> which has been incubated for one week, i.e., Ca<sup>4.6</sup>, P<sup>16.9</sup>.

half of the pulp was suspended in the usual *Sol A* and the other half in *Sol N* (no Ca or P). The extracts were centrifuged immediately. The extracts in *Sol A* averaged  $\text{Ca}^{5.3} \times \text{P}^{13} = 68.9$ , while those in *Sol N* averaged  $\text{Ca}^{4.1} \times \text{P}^{16.1} = 66$ . In this brief interval the pulp had practically saturated *Sol N* with these elements. P exceeding Ca by approximately 4 times. When employed in serum media of the type illustrated in Table II, the extracts in *Sol N* produced more rapid calcification than those in *Sol A*. Calculation shows that this was to have been expected from the high Ca x P values produced upon mixing the high Ca of the serum and salt solutions with tissue extracts having P values around 16.

Correct Ca and P ratios (but high products) could be produced in fresh extracts by diluting the pulp in balanced salt solution containing elevated Ca and no P. Upon incubation, however, these extracts precipitated excessively. Calculation indicated that this modification was impractical, since each mg% of additional P liberated by phosphatases must now be multiplied by a higher initial level of Ca.

Since P is dialyzable, correct values and ratios for Ca and P should be obtained by dialysis of embryo juice. A fresh embryo extract containing  $\text{P}^{10.7}$  was diluted with 2 volumes of balanced salt solution containing  $\text{Ca}^7\text{P}^9$ , to produce an anticipated value of  $\text{Ca}^{6.5}$  and  $\text{P}^{3.6}$ . Upon being rapidly concentrated to the original volume in a pressure dialyzer modified after Simms,<sup>9</sup> the first half of the filtrate contained 3.5 mg% of P, the second half 3.6, and the residue in the bag 3.7 mg% of P. Though this procedure can produce embryo juice of any desired P value, it is not practical as a routine and also washes out small molecules which are important to nutrition.

*Pasteurization to inactivate phosphatases.* Inactivation of phosphatases by pasteurizing the embryo juice at 60° for 30 minutes, which causes some coagulation of protein, reduced the initial P values by 1-2 mg% and the Ca x P products by approximately 6 to 12. The P values did not rise on incubation of this juice alone and rose only slowly during the incubation with serum, presumably due

<sup>9</sup> Simms, H. S., and Stillman, N. P., *J. Gen. Physiol.*, 1937, III, 20, 649.

TABLE I.  
Effect of Age of Pooled Embryos on the Ca and P Values of Fresh EM<sub>30</sub>.

Pool No.	Incubation of eggs (days)	Avg wt* of embryos (g)	Ca (mg %)	P (mg %)	Ca x P	Ratio Ca : P
1	8	1.0	4.0	7.4	29.6	1: 1.9
2	10	1.5	4.1	10.2	41.8	1: 2.5
3†	11	1.9†	6.2†	8.7†	53.9†	1: 1.4†
4	11	2.3	5.4	12.3	66.4	1: 2.3
5	11	2.4	5.2	13.1	68.1	1: 2.5
6	11	2.6	5.5	12.9	71.0	1: 2.3
7	12	3.0	7.0	14.9	104.2	1: 2.1
Avg values for No. 4-6 (11-day Em)		2.4	5.4	12.8	68.5	1: 2.4

\* These eggs were much smaller than American eggs.

† Unusual Ca and P values and not averaged with No. 4-6; the only 11-day EM studied which failed to cause calcification when used at 15% levels.

tenance of human fibroblasts. It was decided to continue the work with extracts prepared from 11-day embryos.

*Varying proportions of serum and embryo juice.* As may be seen in Table II, the use of varying proportions of 50% serum and 30% embryo juice in a series of experiments revealed that *PLS<sub>17</sub>EM<sub>20</sub>* usually produced calcification after a second application (following one renewal): *PLS<sub>25</sub>EM<sub>15</sub>* ordinarily permitted 3 applications; *PLS<sub>33</sub>EM<sub>10</sub>* was tolerated for 4 or sometimes 5 applications, while *PLS<sub>42</sub>EM<sub>5</sub>* could be employed during intervals up to 8 weeks. The latter medium was the only one which assured that long-term cultures would not be lost due to calcification during repeated transplantation. In it the explants or transplants sometimes turned opaque but the precipitate did not spread among the growing cells.

From the Ca and P data in the table it is apparent that the media with higher proportions of embryo juice contained P in excess of Ca. Furthermore, the higher Ca x P values occur where there is the least serum protein to stabilize these compounds in solution.<sup>8</sup> It was only when the embryo juice was reduced to 5% that Ca was appreciably in excess of P and the "potential" Ca x P could be expected to remain below 60.

Since P is liberated during incubation of the cultures, and both Ca and P are absorbed in the plasma and the explants, these potential

values could not be determined without destruction and analysis of cultures being maintained for another purpose. Evidence that fresh embryo juice is saturated with respect to Ca and P and that active phosphatases liberate still more P during incubation will be presented in justification of the calculations employed.

*Embryo juice as a solution saturated with Ca and P.* Though the actual levels of Ca and P tended to vary in different lots of extract from 11-day embryos, these values were usually related inversely to each other and showed fairly good correlation between the Ca x P products and the average weights of the embryos employed. The relatively clear extracts were observed to become more opalescent or actually turbid during refrigeration. When incubated at 37° for a few days, they quickly passed through a cycle of opalescence, turbidity and sedimentation. In 9 extracts which were allowed to pass through this cycle for 3 to 5 days at 37° the average initial values of  $Ca^{5.3} \times P^{12.6} = 66.8$  had changed to  $Ca^{4.2} \times P^{16.1} = 67.6$  after incubation. These products indicate that the fresh embryo juice was saturated. The increase in P had been more than the indicated 28%, since some had been involved with the Ca precipitated.

Other evidence of saturation appeared upon trying to suspend fresh embryo pulp in salt solutions designed to lower or modify the levels of Ca and P. 4 batches of 11-day embryos were pulped without diluent. One

<sup>8</sup> Rosenheim, A. H., *Biochem. J.*, 1934, **28**, 699.

TABLE IV.

Data for Estimation of Initial Ca and P Products of Media Which Combine Human Serum with Chick or Beef Embryo Juice.

	Placental serum			EM <sub>50</sub> , chick (11-day embryos)			EM <sub>50</sub> , beef* (4-inch embryos)			Balanced salt solution		
	%	Ca	P	%	Ca	P	%	Ca	P	%	Ca	P
1.	100	12.0	5.5	50	4.3	13.9	50	10.5	12.0	100	5.0	2.4
2.				15†	1.3	4.2	15†	3.2	3.6			
				10	0.9	2.8	10	2.1	2.4			
	60	7.2	3.3	6†	0.5	1.7	6†	1.3	1.4	28	1.4	0.7
	50	6.0	2.8	5	0.4	1.4	5	1.1	1.2	40	2.0	1.0
	40	4.8	2.2	4	0.3	1.1	4	0.8	1.0	52	2.6	1.3
	30	3.6	1.7	3	0.3	0.8	3	0.6	0.7	64	3.2	1.5
3.	20	2.4	1.1	2	0.2	0.6	2	0.4	0.5	76	3.8	1.8
	10	1.2	0.6	1	0.1	0.3	1	0.2	0.2	88	4.4	2.1

1. Ca and P of constituents employed.

2. These concentrations of embryo juice, though satisfactory with chicken serum, are not recommended in the presence of mammalian sera.

3. If using 10:1 ratios of serum and embryo juice simply add the values on appropriate horizontal line, using data for the embryo juice employed. Otherwise add the values for the actual concentrations being used.

## Example

	%	Ca	P	Ca x P
PLS <sub>40</sub>	40	4.8	2.2	
EM <sub>4</sub> (beef)	8	0.8	1.0	
Salt sol	52	2.6	1.3	
		8.2	4.5	= 36.9

\* Data from Dr. Geo. O. Gey, extract prepared in salt solution with Ca<sup>9.9</sup> and P<sup>3.6</sup>.

† Values take into account the fact that 50% extracts occupy twice the volume indicated by the percentage concentrations.

tential" Ca x P will exceed 60. The suitability of such formulae presumably depends on the fact that the increased concentration of serum protein raises the levels of Ca x P which can be kept in solution.<sup>8</sup>

**Discussion.** The calcification of tissue cultures in mammalian sera as here described should not be confused with the Liesegang rings of calcium salts which were observed in "aerobic" chick embryo cultures by Hueper and Russell<sup>10</sup> and others to whom they refer. Calcification does not occur in chick cell cultures at appropriate pH, even in high concentrations of embryo juice and in the presence of 40% oxygen. The phenomenon of Hueper and Russell<sup>10</sup> must be attributed to excessive alkalization in cotton stoppered vessels.

Since mixtures of embryo juice and chicken serum may become highly opalescent without inducing calcification, the difference between chicken serum and the mammalian sera may be related in part to the unusual capacity of

chicken serum to carry high Ca and P values.<sup>11</sup> Further indication of differences between chick and mammalian systems is afforded by the observations of Fell and Robinson<sup>12</sup> on the resistance of the chick femur to calcification.

The calcification of mammalian tissue cultures in the presence of mammalian sera under similar circumstances is explained primarily by the high P values contributed by embryo juice and by the lower levels of bone salt required for saturation, while the differences between the 3 mammalian sera are doubtless related to differences in the natural levels of total and diffusible calcium.<sup>13</sup>

It would appear that cultivated mammalian tissues may differ in their phosphatase activity *in vitro*. With rat tissues in the media here described the plasma tended to remain clear

<sup>11</sup> Greenberg, D. M., Lawson, C. E., Pearson, P. B., and Burmester, B. R., *Poultry Science*, 1936, 15, 483.

<sup>12</sup> Fell, H. B., and Robinson, R., *Biochem. J.*, 1934, 28, 2243.

<sup>13</sup> Schmidt, C. A., and Greenberg, D. M., *Physiol. Rev.*, 1935, 15, 297.

<sup>10</sup> Hueper, W. C., and Russell, M. A., *Am. J. Med. Sciences*, 1933, 180, 383.

to serum phosphatase. These extracts, when used in  $PLS_{25}EMP_{15}$  induced good growth and permitted continuous maintenance of several series of cultures in which all of the control cultures in unheated embryo juice were lost by calcification. The pasteurization of embryo juice has previously been shown to inhibit the liberation of free lipid and to promote greater longevity of chick embryo cultures than is possible with fresh embryo juice.<sup>5</sup>

*Calcification studies in plasma blanks with 4 types of serum.* Though growing cell colonies obviously play a role in phosphate liberation and CaP deposition near the central explants, blank tests in the plasma base with serum-embryo juice mixtures renewed at weekly intervals provided a convenient method for comparing different degrees of supersaturation, for example in systems containing serum from different animal species. These experiments were undertaken because Carrel had warned against the opacity which develops promptly if rabbit serum is employed in tissue cultures, because our attempts to substitute bovine for human serum had always increased calcification, and because the embryo juices referred to in this study had not been causing calcification in chick embryo tissue cultures.

The data in Table III summarize the observations on 3 batches of embryo juice in combination with 4 animal sera. Though the human and bovine sera gave comparable results in 2 of the trials, it is evident from the summarized scores, together with the experience in tissue cultures, that the 4 sera may be arranged as follows with respect to calcification: rabbit > bovine > human > chicken.

During these tests in the absence of cell colonies it became evident that a slowly increasing opalescence of the supernatant media containing mammalian sera provided the most favorable circumstance for calcification of the plasma layer, but that chicken serum media could become highly opalescent without inducing calcification. When incubated in plain glass tubes for several weeks, both rabbit and chicken serum media liberated more free lipid (which appeared as a scum

TABLE III.  
Calcifying Tendency of Serum from 3 Mammalian Species and from Chickens, as Revealed in Plasma Blanks.

Embryo juice No.	Opacity in plasma following weekly renewal with serum 25% and embryo juice 15% (sera)			
	Rabbit	Bovine	Human	Chicken
63	034	012	012	000
67	044	023	023	000
70	144	023	002	001
Total scores*	222	58	37	1

\* The 3 values for each serum represent the opacity rating on a plus scale at the end of the first, second and third weeks of the experiment. For example, 034 indicates no opacity after 1 week, 3+ opacity after 2 weeks and 4+ after 3 weeks. To illustrate the difference between the sera, these values are simply added as a total "score."

on the surface) than the bovine or human sera. The relation of free lipid to lipid accumulation in cells has been emphasized elsewhere.<sup>5</sup>

*Preparation of media with acceptable Ca and P content.* The data in Table IV provide a basis for estimating the total Ca and P content of media composed of human placental serum and chick or beef embryo juice. The ingredients differ from those used at Cullin. The salt solution (prior to the addition of buffer) contains only 5 mg% of Ca, the approximate concentration of diffusible Ca in human serum. When mixed with any proportion of serum it provides ionized Ca levels similar to those in body fluids of corresponding protein content. Both chick and beef embryo juice should be prepared at 50% concentrations. Dilute extracts will tend to be saturated with Ca and P. If such extracts comprise a large portion of the final medium, it is impossible to dilute or modify their inorganic constituents to appropriate levels. When embryo juice is employed in concentrations greater than 5%, it is not necessary or desirable to incorporate P in the salt solution used for diluting the medium.

Since 10:1 ratios of serum and embryo juice seem to be optimal for good growth and quality of mammalian cells, the examples in the table are based on these ratios. With formulae such as  $PLS_{60}EM_6$  the initial Ca x P may be nearly 55, while the "po-

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17182. Stimulation of Gastric Secretion in Man by Theophylline Ethylenediamine.

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Roth and Ivy<sup>1-3</sup> reported that caffeine parenterally or intragastrically, stimulates gastric secretion in cat and man. They noted no effect when caffeine was given alone in the dog. Robertson and Ivy<sup>4</sup> observed that caffeine and theophylline potentiate the effect of histamine on gastric secretion in dogs, but did not stimulate when given alone. Wood<sup>5</sup> noted that in some cats theophylline and theobromine alone stimulated gastric secretion. No reference to the effect of theophylline on gastric secretion in man could be found in the literature, and so the present study was undertaken.

**Method.** After a 12 hour fast, a Levine tube was introduced into the stomach and the

contents aspirated at 15 minute intervals. After the fourth sample was withdrawn, 0.5 g of theophylline ethylenediamine was administered slowly intravenously dissolved in 10 cc of physiological saline, or through the stomach tube in 200 cc of water. The gastric contents were aspirated at 15 minute intervals thereafter for 90 minutes except that with the orally administered drug the first period after introduction of the drug into the stomach was 20 minutes in length instead of 15 minutes. For each sample the total milligrams of free acid was determined. Ten subjects received the theophylline intravenously and ten received the theophylline through the stomach tube.

**Results and comment.** The results obtained with intravenous administration are shown in Fig. 1, with oral administration in Fig. 2. The maximum effect of theophylline is noted 15 minutes after intravenous administration, whereas with the oral preparation the maximum effect occurred 45 minutes after administration.

These results indicate that theophylline is

<sup>1</sup> Roth, J. A., and Ivy, A. C., *Am. J. Physiol.*, 1944, 141, 454.

<sup>2</sup> Roth, J. A., and Ivy, A. C., *Gastroenterology*, 1944, 2, 274.

<sup>3</sup> Roth, J. A., and Ivy, A. C., *Am. J. Physiol.*, 1944, 142, 107.

<sup>4</sup> Robertson, C., and Ivy, A. C., *Fed. Proc.*, 1949, 8, 133.

<sup>5</sup> Wood, D. R., *British Med. J.*, 1948, 2, 283.

in the region of the cell colonies and to become opaque elsewhere. Similar observations have been recorded by Earle.<sup>1</sup>

In the presence of human tissues the freshness and potential phosphatase activity of the embryo juice influenced the type of calcification. If embryo juice was mixed with undiluted serum during refrigeration, the precipitation cycle and liberation of P was inhibited. The media subsequently prepared possessed excellent growth stimulating properties and the cell colonies appeared to play a role in phosphatase activation, since calcification centered around the transplants and among the growing cells. If, on the other hand, media were prepared from incubated juices which had passed through the precipitation cycle, the growth promoting properties were greatly diminished and calcification occurred diffusely throughout the plasma as though resulting from ionic exchange and simple supersaturation.

The present suggestions concerning the desirability of keeping the Ca x P products below 60 and the serum and embryo juice ratios at 10:1 serve only as an approximation of permissible thresholds under average circumstances. The work of Robinson and co-workers<sup>8</sup> reveals the manner in which protein, glucose, magnesium and the serum from different animal species influences the *in vitro* calcification of cartilage. From these and other data on the solubility of Ca and P in body fluids,<sup>14,15</sup> it is apparent that the permissible Ca x P will be related to the concentration of serum protein in the medium.

Several precautions should be borne in mind during the preparation of embryo juices and of serum. These include: (1) the use of 10- or 11-day chick embryos and exclusion of any which appear over-age; (2) the selection of beef embryos which do not exceed 4 inches in length; (3) the preparation and storage of extracts at 50% concentrations so that the final dilution will permit modification of the Ca and P to desired levels; (4) the maintenance of the lowest practical temperatures

during extraction and centrifugation; (5) storage of the extracts in a frozen state. The loss of protein during pasteurization of 50% juices is excessive. If this procedure is employed to inactivate phosphatases, the juice should be diluted with an equal volume of salt solution just prior to heating. Hemolysis of red cells during serum production must be expected to elevate the P values<sup>7</sup> while filter pads which liberate magnesium, alkali, or other inorganic radicals should be avoided.

When growth inhibition or plasma opacity are shown to be due to calcification (as evidenced by darkening of the central transplants, by data on the Ca and P values of the medium, or by solubility of the opacity in N/10 HCl) the interim steps which should be taken are: (a) rapid transplantation with exclusion of the central portion of the colony or (b) tryptic digestion and removal of the opaque plasma if the colonies are to be retained in the original vessels. Re-evaluation and correction of the inorganic composition of the medium is necessary to prevent recurrence of the difficulty.

*Summary and conclusions.* 1. Chick embryo juice, in concentrations suitable for chick cell cultures, induces calcification of the cell colonies and plasma in mammalian cell cultures.

2. Fresh embryo pulp and extract are saturated with Ca and P. Ca' and particularly P increase with age of the embryos, their ratios being 1:2.4 at 11 days. Upon incubation the P increases an additional 30%.

3. Pasteurization of embryo extracts inactivates the phosphatases and prevents calcification in cell cultures receiving high concentrations of embryo juice.

4. Calcification of cultures in the presence of mammalian sera is due to combining the Ca of serum and salt solution with the high levels of P in embryo juice, to the further liberation of P during incubation, and to the limited Ca x P products held in solution by mammalian sera.

5. The calcifying propensity of chicken serum is much less than that of 3 mammalian sera, which may be arranged as follows: human < bovine < rabbit.

Received May 18, 1949. P.S.E.B.M., 1949, 71.

<sup>14</sup> Peters, J. P., and Eiserson, L., *J. Biol. Chem.*, 1929, **84**, 155.

<sup>15</sup> McLean, F. C., and Hastings, A. B., *J. Biol. Chem.*, 1935, **108**, 285.

some years recognized that certain varieties of acetic acid bacteria, now known as *Acetobacter viscosum* and *Acetobacter capsulatum*, often are associated with the type of spoilage of beer known as "ropiness." Recently Shimwell<sup>12,13</sup> has shown that the production of ropiness by these bacteria depends upon their capacity to form slime from dextrin, a natural constituent of beer. This author found that cultures of *A. viscosum* and of *A. capsulatum* became grossly viscous in dextrin-rich beer or in a medium of yeast extract containing 4 per cent dextrin, but not in beer devoid of dextrin or in yeast extract media in which the dextrin was omitted or replaced by glucose, fructose, maltose, or sucrose. It is of historical interest that Henneberg<sup>14</sup> fifty years previously had reported slime formation in dextrin-rich media (also often in beer) as a differential feature of the related *Bacterium (Acetobacter) induratum*.

Little has been recorded of the nature of the slimy material produced by the acetic acid bacteria associated with ropy beer. Hampshire<sup>8</sup> isolated a mucilaginous material from beer made ropy by inoculation with *Acetobacter* R, a variety now said to be closely related to *A. viscosum*.<sup>15</sup> This material was ash free, contained approximately 1% nitrogen, and on acid hydrolysis yielded 89.5% reducing sugar, calculated as glucose. Polarimetric readings were stated not to agree with this figure, but nevertheless the author concluded that the material was "of the nature

of a dextran", i.e., a polyglucoside. In the case of the slimy materials known to have been produced from dextrin by *A. viscosum* and *A. capsulatum* we were unable to find any published information apart from the statement of Shimwell<sup>12</sup> that these must be of carbohydrate nature because of their origin.

The present study was made with two strains of *Acetobacter*, procured from the National Collection of Type Cultures, capable of producing a gelatinous type of growth in media containing dextrin: i.e., *A. viscosum* 7216, isolated from a ropy top fermentation of beer by Tosic, and *A. capsulatum* 4943, isolated from ropy beer by Shimwell.<sup>9,12</sup> The capacity of these strains to produce material serologically like dextran was established by experiments made in comparison with a strain (B) of *Leuconostoc mesenteroides* originally used in the establishment of the serological reactivity of sucrose-derived dextrans.<sup>1</sup>

The utilization of dextrin as the substrate for the formation of serologically reactive material by the *Acetobacter* cultures, in contrast to the utilization of sucrose for the synthesis of serologically similar material by the *Leuconostoc mesenteroides* can best be illustrated by the following experiment. The *Acetobacter* and *Leuconostoc* strains, after preliminary passage in glucose broth, were inoculated into a series of tubes of broth consisting of 0.5% Difco yeast extract plus 5% of one of a number of different carbohydrates, including dextrin and sucrose. The dextrin was of bacteriological grade, that is, a starch-free, alcohol-precipitated product; the sucrose was a sample of beet sugar known to be free of the traces of material precipitating with dextran-reactive antisera which occur in most lots of reagent and commercial sucrose;<sup>15,16</sup> all the other sugars were of reagent grade. After incubation at 25°C for one week, the cultures which had grown were centrifuged, and their neutralized supernatant fluids tested at several dilutions for capacity to give precipitation with each of two different dextran-

<sup>6</sup> Baker, J. L., Day, F. E., and Hulton, H. F. E., *J. Inst. Brewing*, 1912, **18**, 651.

<sup>7</sup> Day, F. E., and Baker, J. L., *Cent. f. Bakt., Abt. 2*, 1913, **36**, 433.

<sup>8</sup> Hampshire, P., *Bull. Bureau of Bio-Technology*, 1922, **1**, 179, 199.

<sup>9</sup> Shimwell, J. L., *J. Inst. Brewing*, 1936, **42**, 555.

<sup>10</sup> Comrie, A. D., *J. Inst. Brewing*, 1939, **45**, 342.

<sup>11</sup> Walker, T. K., and Tosic, J., *J. Inst. Brewing*, 1945, **51**, 245.

<sup>12</sup> Shimwell, J. L., *J. Inst. Brewing*, 1947, **53**, 250.

<sup>13</sup> Shimwell, J. L., *Wallerstein Labs. Commun.*, 1948, **11**, 27.

<sup>14</sup> Henneberg, W., *Cent. f. Bakt., Abt. 2*, 1898, **4**, 933 (Abstract).

<sup>15</sup> Neill, J. M., Hehre, E. J., Sugg, J. Y., and Jaffe, E., *J. Exp. Med.*, 1939, **70**, 427.

<sup>16</sup> Neill, J. M., Sugg, J. Y., Hehre, E. J., and Jaffe, E., *Am. J. Hyg.*, 1941, **343**, 65.



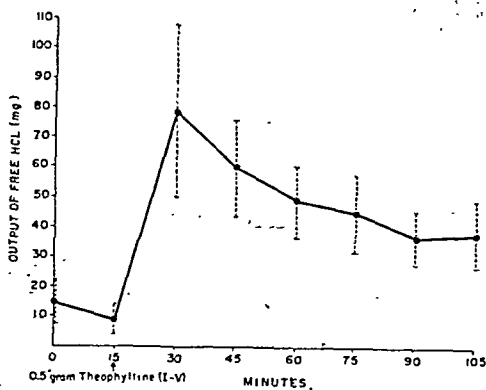


FIG. 1.

Stimulation of free HCl output by intravenous administration of theophylline ethylenediamine. Each dot represents the mean value of determinations on 10 subjects. The vertical bars represent the standard errors of the respective means.

similar to caffeine in its effect on gastric secretion in man.

In the course of the study 3 patients received a tablet form of theophylline with variable response, and 4 patients received theobromine orally with no gastric stimulation.

No decision can be made as to whether the

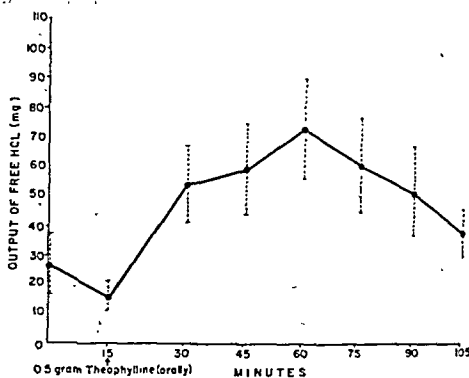


FIG. 2.

Stimulation of free HCl output by oral administration of theophylline ethylenediamine. See legend of Fig. 1.

stimulation produced by orally administered aminophylline is due to direct local action or to an action after absorption.

**Summary.** Theophylline stimulates the secretion of acid gastric juice in man on oral or intravenous administration.

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### 17183. Bacterial Conversion of Dextrin into a Polysaccharide with the Serological Properties of Dextran.

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The purpose of the present paper is to report the capacity of certain acetic acid bacteria, and of cell-free solutions of enzymes obtained from them, to synthesize from dextrin material with serological properties like those which we have earlier described for dextran.<sup>1</sup> Hitherto, the only enzymatic pathway known for the synthesis of dextran was that from sucrose.<sup>2,3</sup> We know of no report of interconversion of material of the starch-

glycogen class and dextran, although both possess related chemical structures and can indeed be synthesized from the same substrate (sucrose) by appropriate bacterial enzyme systems.<sup>2-5</sup> Ultimate proof of the biological synthesis of dextran from dextrin must of course rest upon chemical studies, which are now in progress. However, the unequivocal nature of the present serological data leaves little doubt that such a conversion does in fact occur.

British brewing bacteriologists<sup>6-11</sup> have for

<sup>1</sup> Sugg, J. Y., and Hehre, E. J., *J. Immunol.*, 1942, **43**, 119.

<sup>2</sup> Hehre, E. J., and Sugg, J. Y., *J. Exp. Med.*, 1942, **75**, 339.

<sup>3</sup> Hehre, E. J., *J. Biol. Chem.*, 1946, **163**, 221.

<sup>4</sup> Hehre, E. J., and Hamilton, D. M., *J. Bact.*, 1948, **55**, 197.

<sup>5</sup> Hehre, E. J., *J. Biol. Chem.*, 1949, **177**, 267.

some years recognized that certain varieties of acetic acid bacteria, now known as *Acetobacter viscosum* and *Acetobacter capsulatum*, often are associated with the type of spoilage of beer known as "ropiness." Recently Shimwell<sup>12,13</sup> has shown that the production of ropiness by these bacteria depends upon their capacity to form slime from dextrin, a natural constituent of beer. This author found that cultures of *A. viscosum* and of *A. capsulatum* became grossly viscous in dextrin-rich beer or in a medium of yeast extract containing 4 per cent dextrin, but not in beer devoid of dextrin or in yeast extract media in which the dextrin was omitted or replaced by glucose, fructose, maltose, or sucrose. It is of historical interest that Henneberg<sup>14</sup> fifty years previously had reported slime formation in dextrin-rich media (also often in beer) as a differential feature of the related *Bacterium* (*Acetobacter*) *industrium*.

Little has been recorded of the nature of the slimy material produced by the acetic acid bacteria associated with ropy beer. Hampshire<sup>5</sup> isolated a mucilaginous material from beer made ropy by inoculation with *Acetobacter* R, a variety now said to be closely related to *A. viscosum*.<sup>13</sup> This material was ash free, contained approximately 1% nitrogen, and on acid hydrolysis yielded 89.5% reducing sugar, calculated as glucose. Polarimetric readings were stated not to agree with this figure, but nevertheless the author concluded that the material was "of the nature

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The present study was made with two strains of *Acetobacter*, procured from the National Collection of Type Cultures, capable of producing a gelatinous type of growth in media containing dextrin: i.e., *A. viscosum* 7216, isolated from a ropy top fermentation of beer by Tosic, and *A. capsulatum* 4943, isolated from ropy beer by Shimwell.<sup>9,12</sup> The capacity of these strains to produce material serologically like dextran was established by experiments made in comparison with a strain (B) of *Leuconostoc mesenteroides* originally used in the establishment of the serological reactivity of sucrose-derived dextrans.<sup>1</sup>

The utilization of dextrin as the substrate for the formation of serologically reactive material by the *Acetobacter* cultures, in contrast to the utilization of sucrose for the synthesis of serologically similar material by the *Leuconostoc mesenteroides* can best be illustrated by the following experiment. The *Acetobacter* and *Leuconostoc* strains, after preliminary passage in glucose broth, were inoculated into a series of tubes of broth consisting of 0.5% Difco yeast extract plus 5% of one of a number of different carbohydrates, including dextrin and sucrose. The dextrin was of bacteriological grade, that is, a starch-free, alcohol-precipitated product; the sucrose was a sample of beet sugar known to be free of the traces of material precipitating with dextran-reactive antisera which occur in most lots of reagent and commercial sucrose,<sup>15,16</sup> all the other sugars were of reagent grade. After incubation at 25°C for one week, the cultures which had grown were centrifuged, and their neutralized supernatant fluids tested at several dilutions for capacity to give precipitation with each of two different dextran-

<sup>6</sup> Baker, J. L., Day, F. E., and Hulton, H. F. E., *J. Inst. Brewing*, 1912, 18, 651.

<sup>7</sup> Day, F. E., and Baker, J. L., *Cent. f. Bakt., Abt. 2*, 1913, 36, 433.

<sup>8</sup> Hampshire, P., *Bull. Bureau of Bio-Technology*, 1922, 1, 179, 199.

<sup>9</sup> Shimwell, J. L., *J. Inst. Brewing*, 1936, 42, 585.

<sup>10</sup> Comrie, A. D., *J. Inst. Brewing*, 1939, 45, 342.

<sup>11</sup> Walker, T. K., and Tosic, J., *J. Inst. Brewing*, 1945, 51, 245.

<sup>12</sup> Shimwell, J. L., *J. Inst. Brewing*, 1947, 53, 280.

<sup>13</sup> Shimwell, J. L., *Wallerstein Labs. Commun.*, 1948, 11, 27.

<sup>14</sup> Henneberg, W., *Cent. f. Bakt., Abt. 2*, 1898, 4, 933 (Abstract).

<sup>15</sup> Neill, J. M., Hehre, E. J., Sugg, J. Y., and Jaffe, E., *J. Exp. Med.*, 1939, 70, 427.

<sup>16</sup> Neill, J. M., Sugg, J. Y., Hehre, E. J., and Jaffe, E., *Am. J. Hyg.*, 1941, 34B, 65.

TABLE I.

Capacities to Precipitate with Dextran-reactive Antiserums Shown by the Culture Fluids of *Acetobacter* and *Leuconostoc* Grown with Different Carbohydrates.

Strain	Highest dilution of the fluids from cultures with different carbohydrates that gave precipitation with type 2 and with type 20 pneumococcus antiserums*				
	Dextrin	Maltose	Sucrose	Raffinose	Other sugars†
<i>Acetobacter viscosum</i> , 7216	50,000	10‡	0	0	0
" <i>capsulatum</i> , 4943	50,000	0‡	0	0	0
<i>Leuconostoc mesenteroides</i> , B	0	0	100,000	20	0

\* 0 = no precipitation with either antiserum in tests with 1:10 or 1:100 dilutions of the culture fluids.

† Glucose, fructose, mannose, galactose, sorbose, xylose, arabinose, mannitol, inositol, trehalose,  $\alpha$ -methylglucoside, lactose, melibiose, or inulin.

‡ Fluids from cultures grown with a different sample of commercial "C. P." maltose gave reactions at dilutions as high as 1:400 with the dextran-reactive antiserums.

reactive antiserums; these were a type 2 pneumococcus antiserum (1:50 dilution) and a type 20 pneumococcus antiserum (1:18 dilution) known to give precipitation with purified *Leuconostoc mesenteroides* strain B dextran in dilutions as high as 1:4 million. Mixtures of 0.2 ml of diluted culture fluid and 0.2 ml of diluted antiserum in clear 10 x 75 mm test tubes were incubated at 37°C for 1 hour and then observed for the development of cloudiness or precipitation.

As shown in Table I, large amounts of material reactive with the type 2 and the type 20 pneumococcus antiserums were present in the fluids of the *A. viscosum* and *A. capsulatum* cultures grown with dextrin, while with one exception, none was detected in the fluids of the cultures grown with other carbohydrates. Traces of reactive material were found in the fluids of the cultures with maltose, but we believe these can be accounted for by the presence of minute amounts of dextrin in the maltose. A large amount of material (dextran) reactive with the same antiserums was produced from sucrose by the *Leuconostoc* culture, and a trace also from raffinose, but none was produced from the dextrin or from any of the other sugars tested.

More complete information on the nature of the serologically reactive substance produced by *A. capsulatum* 4943 has been obtained by studies made with material isolated from a dextrin broth culture. Several liters of viscous culture fluid yielded an amount (approximately 15 g) of serologically reactive polysaccharide which corresponded to more

than 20% of the weight of the dextrin used in the medium. An account of the separation, purification, and chemical properties of the *A. capsulatum* polysaccharide will be published in a subsequent report. Results of the serological precipitation tests which we have made with this material in comparison with purified dextran isolated from a sucrose broth culture of *Leuconostoc mesenteroides* B<sup>1</sup> are shown in Table II.

Both the *A. capsulatum* polysaccharide and the *L. mesenteroides* dextran gave visible precipitation with type 2 and type 20 pneumococcus antiserums in dilutions from 1:10,000 to 1:4 million; both gave precipitation also with type 12 pneumococcus antiserum though only at much lower dilutions; and both failed to react at 1:10,000 or higher dilutions with any of the control serums, which included high-titered antipneumococcal serums of several types, normal serums from rabbits which furnished type 2 and 20 serums, and an antiserum capable of precipitating with high dilutions of the polyfructoside levan. The mutual cross-reactions with the types 2, 20 and 12 pneumococcus antiserums, given by the *A. capsulatum* 4943 and *L. mesenteroides* B polysaccharides, and especially the comparably high degree of reactivity versus the types 2 and 20 in comparison to the lower degree of reactivity versus the type 12 are evidences of an extraordinarily close serological likeness between these two materials. Additional evidence for the close serological similarity, not presented in Table II, was that absorption of type 2 pneumococcus anti-

TABLE II.

Serological Similarity of the Dextrin-derived Polysaccharide of *Acetobacter capsulatum* to the Sucrose-derived Dextran of *Leuconostoc mesenteroides*.

Kind of serum	Dilution of serum	Highest dilution of antigen that gave precipitation*	
		<i>Acetobacter capsulatum</i> polysaccharide	<i>Leuconostoc mesenteroides</i> dextran
Antipneumococcus, type 2 (Led) †	1:50	4,000,000	4,000,000
" " 2 (rabbit S5)	1:25	2,000,000	2,000,000
" " 20 ( " 02)	"	4,000,000	4,000,000
" " 12	"	50,000	10,000
" " 1, 3, 5, 8, 14 (Led) †	1:25, 1:50	0	0
Normal, pre-immunization (rabbits S5, 02)	1:12, 1:25	0	0
Levan-reactive, <i>Bacillus</i> N9	1:12	0	0

\* 0 = no precipitation with 1:10,000 or any higher dilution of the polysaccharide.

† Refined, or concentrated, rabbit antisera kindly supplied by Dr. H. D. Piersma, Lederle Laboratories, Pearl River, N.Y.

serum with either polysaccharide removed the antibodies reactive with both polysaccharides.

The enzyme system in *A. capsulatum* responsible for the conversion of dextrin into material serologically like dextran is readily obtainable in solution, free from bacterial cells. Using the fluids from glucose broth cultures as starting material, we have been able to prepare potent concentrates by a method previously described.<sup>5</sup> When "bacteriological" dextrin, Lintner's soluble starch, or certain hydrolysates made either from amylopectin or from crystalline amylose are incubated with such enzyme preparations, amylase-resistant polysaccharide material that gives serological precipitation with dextran-reactive antisera is formed. These results suggest that the action of the *Acetobacter* enzyme is to transfer 1:4 linked glucose units to new polysaccharide material in which the units are linked in some

other fashion, very likely 1:6. Although proof of the exact mechanism of the reaction must await the results of systematic chemical study, recognition of the enzymatic convertibility of amylaceous to non-amylaceous polysaccharide by itself is of biological significance.

**Summary.** Cultures of certain acetic acid bacteria from "ropy" beer, when grown with dextrin but not when grown with other common carbohydrates, produce abundant amounts of polysaccharide material which has serological properties like those of the dextran which is produced from sucrose by *Leuconostoc mesenteroides*. A cell-free enzyme system, capable of causing the conversion of dextrin to material serologically like dextran, has been obtained from one of the cultures.

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## 17184. Anoxic Diversion of the Renal Cortical Blood Flow.

K. J. FRANKLIN, L. E. MCGEE, AND E. ULLMANN. (Introduced by J. F. Fulton.)

From the Department of Physiology, St. Bartholomew's Hospital Medical College.

During 1947-8 Trueta, Franklin, and Amoroso laid plans for the joint solution of various renal problems, and the research to be described, though in fact of more fortuitous origin, is to be regarded as part of that cooperative effort. Briefly, it was thought

probable that the renal cortical blood flow is diverted whenever more important, or temporarily more important, organs are rendered too anoxic. Experiments were, therefore, begun with a view to testing this hypothesis, and in those to be described the whole body was

subjected to anoxia in the expectation that some parts would be more sensitive than the renal cortex, and that in consequence the latter would be, reflexly or otherwise, deprived of some or all of its blood supply, the portion of the total circulating blood volume so diverted being added to the quotas allotted to more needy organs. At the time the work of Toth<sup>1</sup> was not known to the writers; it will be referred to below in the discussion of results.

**Methods.** The animals mainly used were rabbits, anaesthetized by intravenous injection of Nembutal solution, followed by administration of open ether as required; in earlier work Trueta *et al.*<sup>2</sup> found that anaesthesia, maintained by such means for several hours, produced no appreciable change in the renal blood flow. To exclude specific peculiarities, some tests were also made on the cat, dog, rat, and mouse.

Anoxia was produced (1) by occlusion of a straight glass cannula tied into the trachea, and (2) by administration of a lethal dose of carbon monoxide (mixture of air and coal gas).

The renal effects were observed either with both kidneys made visible by laparotomy, or with one or both kidneys exteriorized. The left kidney was exteriorized by bringing it out between the lumbar and abdominal musculatures, the right as seemed best according to the individual circumstances. To determine if the renal effects were nervously intermediated, the left kidney was denervated (usually by division of the nerve or nerves accompanying the renal artery, but on occasion by splanchnic section), and the right kidney was left intact. When desired, the state of the renal blood distribution was precisely ascertained by injecting warmed India ink in a proximal direction through a cannula tied into

the distal aorta, the mesenteric artery having first been ligated. The injection was made at a pressure just above that in the aorta, and ligatures previously passed round the renal vessels were tied while the injection was at its height. Thereafter, both kidneys were exsected and placed in fixative for a day or so before being cut open and prepared for subsequent histological section.

In a few cases a 16 mm color film record was made of the anoxia production and its results. Details of the published films are given after the references at the end of this paper.

**Results.** Occlusion of the tracheal cannula did not usually cause an immediate cessation of respiratory efforts; indeed, these might, for a time at least, increase in rate and/or intensity. Within 20 to 60 seconds, as a rule, the occlusion produced first a darkening, then patchy paling, and finally generalized paling (sometimes spectacular in its degree) of the kidney surface, together with diminution in size of the organ and wrinkling, often very marked, of its capsule. The diminution in volume, if one can judge from cubing linear measurements, was of the order of one-sixth; an oncometer was not used as it would have introduced complicating factors. Sections of innervated kidneys removed at the height of the anoxia effects showed anaemia of the cortical portions; in the denervated kidneys there was no such diversion of cortical blood flow, though it was possible to produce it in such organs by intravascular injection of adrenaline. In some of the former cases there was more blood than normal in the medulla; in others less. When re-entry of air into the lungs was allowed to occur, blood rapidly returned to the kidney surface, at first discretely, but almost immediately thereafter all over. In their rapidity, therefore, the renal changes after anoxia were more dramatic than those produced during its development. In contrast to the restoration of color was the disappearance of the surface wrinkling, which often took a minute or two for its completion. The whole tracheal occlusion experiment could be repeated at very short intervals and, at least in the case of the ex-

<sup>1</sup> Toth, L. A., *Am. J. Physiol.*, 1940, **129**, 532.

<sup>2</sup> Trueta, J., Barelay, A. E., Daniel, P. M., Franklin, K. J., and Prichard, M. M. L., *Studies of the renal circulation*, 1947; Franklin, K. J., *Anoxic diversion of the renal cortical blood flow*, Part I, 1948; Ullmann, E., McGee, L. E., and Franklin, K. J., *Anoxic diversion of the renal cortical blood flow*, Part II, 1949. Oxford: Blackwell Scientific Publications, Ltd.

teriorized kidneys, over a total period of several hours. Administration of carbon monoxide, in the dosage employed, produced equally marked diversion of blood from the cortex but the change was irreversible. Two final notes may be added to the above account. In the first place, partial denervation of one kidney, which was effected in a few cases, divided the single organ into a part which responded to anoxia and a part which did not; this was a convenience for some purposes. Secondly, if the stomach was over-full or the uterus seriously enlarged as a result of pregnancy, the kidneys tended to be found partially "shunted," if one may use the American expression for diversion of the cortical blood supply, from the outset.

*Discussion.* Toth's<sup>1</sup> anoxia experiments were carried out in dogs, and paling of the surface of the dog's kidney during diversion of the cortical blood flow is not readily detected because (1) the capsule is relatively thick, and (2) the cortical color changes are masked by superficial vessels which are unconcerned, or little concerned, in the diversion. Further, the findings of Trueta *et al.*, which showed the significance of such cortical diversion as opposed to an overall reduction in renal arterial inflow, were not begun until 1945. In a future paper two of the present writers (L.E.M., E.U.) will give quantitative details about the degrees of anoxia and of hypercapnia which are required for the production of the renal circulatory changes, and will describe the fluctuations of arterial blood pressure re-

corded during these changes, together with other features of interest. They may also give evidence about the urine flow as affected by anoxia, and offer a possible explanation for the time-lag between the return of color to the kidney surface and the disappearance of the wrinkling from that surface. In this preliminary account, however, the main object is to describe the more obvious features and to point out that the simultaneous use of innervated and denervated kidneys, with the completeness of the denervation shown by a tracheal occlusion test, allows one rapidly to determine if a poison, such as dioxan (see De Navasquez,<sup>3</sup>) or a bacterial toxin, affects the kidneys through nervous intermediation or otherwise. Publication of this present paper is, therefore, a necessary prelude not only to the communication of further finding about anoxia effects, etc., but also to the description of the results of experiments with a number of nephrotoxic substances.

*Summary.* 1. Acute anoxia, sufficient in degree, produces a marked diversion of the renal cortical blood flow in the innervated, but not in the denervated, kidney.

2. Simultaneous study of the reactions of the innervated and denervated kidneys is a simple way of distinguishing the mechanisms of action of different nephrotoxic substances.

<sup>3</sup> De Navasquez, S., *J. Hyg.*, 1936, **35**, 549; De Navasquez, S., *J. Path. Bact.*, 1938, **40**, 47.

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## 17185. Effect of Elevated Body Temperatures on Cryptococcosis in Mice.

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In a study<sup>1</sup> of the effects of temperature on the reproduction and viability of *Cryptococcus neoformans*, the number of viable cells decreased by 85% within 5 days at 103° F

(39.4°C), but increased regularly at 99°F (37.3°C). The writer postulated that differences in body temperatures of laboratory mice and rabbits might account for differences in susceptibility to cryptococcosis. Mice, with rectal temperatures averaging 99.1°F, died

<sup>1</sup> Kuhn, L. R., *Proc. Soc. Exp. Biol. and Med.*, 1939, **41**, 573.

TABLE I.

Survival of Mice Injected with *C. neoformans* and Maintained Either at 35 to 36°C or 24 to 27°C.

Dosage of <i>C. neoformans</i>	Method of inj. and No. injected	Time after injection	No. surviving in mice maintained at temperatures indicated		
			24-27°C	35-36°C	35-36°C after being kept at 24-27°C for time indicated
2 to 2.5 million in 0.5 ml of saline	Intravenous 15 in each temp. group	0 hr	15	15	2 days 15
		48 "	15	11	15
		11 days	0	11	10
		20 "	0	10	6
		30 "	0	6	3
Same	Intraperitoneal 18 in each temp. group	0 hr	18	18	8 days 18
		44 days	0	12	9
		60 "	0	7	4
120 to 150 thousand in 0.03 ml of saline	Intracerebral 25 in each temp. group	0 hr	25	25	2 days 25
		24 "	25	21	25
		10 days	4	15	20
		20 "	0	8	6
		30 "	0	6	2

regularly and relatively rapidly of experimental infections, while rabbits, with a mean temperature of 103.15°F, were affected only rarely and slowly.

To determine if mice could be maintained with elevated body temperatures long enough to study their reaction to cryptococcosis, groups of Swiss mice were kept at various temperatures above 30°C. In still air at 35 to 37°C with 25 to 35% relative humidity, rectal temperatures rose rapidly and remained above 102°F.\* Although a few mice died during an initial day or two of discomfort for all, the majority survived and were in apparent good health at the end of a month, although averaging only 1.3 g gain in weight compared with 7.1 g in room temperature controls. At this time, the rectal temperatures of 2 separate lots of heat-adapted mice averaged 102.8 and 103.4°F, ranging from 101 to 105°F.

Infections with a pathogenic strain† of *C. neoformans* were compared in Swiss mice weighing 17 to 19 g and kept either at 24 to 27°C or at 35 to 36°C. Groups maintained

at each temperature level were injected intravenously, intraperitoneally or intracerebrally with 60-hour Sabouraud agar cultures of *C. neoformans* suspended in 0.85% sodium chloride. Doses for each group as well as the effect of environmental temperatures on survival are shown in Table I. Mice in all groups kept at 35 to 36°C outlived all those maintained at 24 to 27°C. Thus, although all of 15 intravenously injected mice kept at room temperature died of cryptococcosis within 11 days after being infected, only 4 of 15 died during the same period in the higher temperature group. Since these 4 died within 48 hours after injecting and incubating, their deaths cannot be attributed to the slowly developing cryptococcus infection. No other heat-adapted mice died until 19 days after injection. Six survived 30 days without apparent effects.

Although intraperitoneal infections developed more slowly, only 6 of 18 mice kept at 35 to 36°C died during the 44 days following injection. All of the control mice died during the period. In 3 of the 6 heat-adapted mice which died, no cryptococci were found by smear or culture. Seven were still living in apparent health 60 days after being injected.

Intracerebrally injected mice showed less

\* Rectal temperatures were taken with narrow bulb thermometers made by Rascher and Betzold, Inc., Chicago, Ill.

† Army Medical School strain D-2, isolated from a human case at autopsy.

striking differences between the temperature groups. Some kept at 35 to 36°C outlived all the room temperature controls, however.

With each of the series described above, an equal number of mice was similarly injected but kept at 24 to 27°C for 2 or 8 days prior to being moved to the higher temperature. Mice in each of these sets lived longer than those kept only at room temperature, but fewer survived than in the groups placed at 35 to 36°C immediately after injecting (Table I).

Fatal infections in many of the heat-adapted mice may have accompanied body temperatures under the average of 103°F, where cryptococci remain viable or even increase *in vitro*. Also, the temperature of the incubator fell to 33 and 32°C during two overnight periods, and all rectal temperatures taken in the morning were at or below 100.5°F. Rectal temperatures of incubated mice showing signs of infection usually were below 100.5°F and decreased as the animals became moribund.

In the light of the previous *in vitro* studies,<sup>1</sup> the results given above suggest that a direct effect of temperature on the growth and viability of *C. neoformans* should be considered in explaining the degree of natural resistance shown by rabbits.

Inhibition of virus infections in small animals kept at high environmental temperatures has been observed, according to Francis.<sup>2</sup> Coplin and Mills<sup>3</sup> found that white mice which had been adapted to moist heat were *more* susceptible to *Streptococcus hemolyticus* than were mice kept at lower temperatures.

Temperatures of 103°F and above may be inimical to the growth and viability of other

fungi causing systemic mycoses. Textbooks of medical mycology direct that cultures be incubated at both room temperature and at 37° for *Monilia*, *Coccidioides*, *Histoplasma*, *Sporotrichum*, and *Blastomyces* as well as for *C. neoformans*. The writer found<sup>1</sup> that the optimum for rapid growth of *C. neoformans* was approximately 29°C and some workers in this field now consider 28 to 30°C to be optimal for all the pathogenic fungi listed above. It would not be surprising, therefore, if more of these organisms fail to maintain themselves at elevated body temperatures.

It is interesting to consider the possible use of fever therapy, alone or combined with chemotherapy, in treating human cryptococcosis (torulosis). Specific therapy appears to be questionable or even lacking entirely,<sup>4,5</sup> and human cases usually show little or no elevation of body temperature. Cox and Tolhurst<sup>6</sup> discuss the possibilities of hyperthermia in the light of the earlier *in vitro* studies of the writer.

**Summary.** 1. Many mice infected with a pathogenic strain of *Cryptococcus neoformans* and maintained, with elevated body temperatures, at 35 to 36°C, lived longer than all infected mice kept at 24 to 27°C.

2. These and previous *in vitro* studies suggest that a direct effect of temperature should be considered in explaining the degree of natural resistance of rabbits to cryptococcosis.

3. Elevated body temperatures might be useful in assisting treatment of human cryptococcosis.

<sup>4</sup> Cox, Leonard B., and Tolhurst, Jean C., *Human Torulosis*, 1946, pp. 79, 129, Melbourne University Press.

<sup>5</sup> Conant, Norman F., In *Bacterial and Mycotic Infections of Man*. Edited by Dubos, R. J., 1948, p. 600, J. B. Lippincott Co.

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<sup>2</sup> Francis, Thomas, Jr., In *Bacterial and Mycotic Infections of Man*. Edited by Dubos, R. J., 1948, p. 98, J. B. Lippincott Co.

<sup>3</sup> Coplin, J. W., and Mills, C. A., *Science*, 1939, 90, 275.



# 17186. A Virus Isolated from Patients Diagnosed as Non-Paralytic Poliomyelitis or Aseptic Meningitis.\*

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It is the purpose of this report to describe the isolation of a filtrable agent from patients with an illness resembling non-paralytic poliomyelitis which occurred during 1948 in southern New England. The virus was sought following the report by Dalldorf and Sickles,<sup>1</sup> and is apparently similar to that which they obtained from the feces of 2 patients diagnosed as paralytic poliomyelitis. Like their agent, the virus infected newborn albino mice and produced in them weakness and paralysis accompanied by diffuse myositis.

The following aspects of work with this virus will be described:

- (1) recovery of the virus from the feces of patients and its neutralization by their sera;
- (2) description of the experimental disease and some properties of the virus;
- (3) inapparent infection of chimpanzees;
- (4) accidental infection of man;
- (5) occurrence of the virus in "poliomyelitis" patients in Ohio and North Carolina as well as in New England;
- (6) occurrence of the virus in extra-human sources (sewage and flies);
- (7) immunological types of the virus.

(1) *Isolation of virus from patients diagnosed as having non-paralytic poliomyelitis or aseptic meningitis.* During the summer and fall of 1948 samples of feces were collected from 16 representative patients from Connecticut and Rhode Island, 13 of whom presented clinical features consistent with non-paralytic poliomyelitis (Table I), and 3 of whom had definite muscle weakness and were diagnosed as paralytic poliomyelitis. The fecal samples from these 16 patients were tested in monkeys

for poliomyelitis virus and were also examined for the agent infectious for newborn mice.

Fecal samples from 5 (Nos. 1-5) of the 13 patients with non-paralytic illnesses produced disease in newborn mice, but not in monkeys. From one to 4 rhesus monkeys (*Macaca mulatta*) were used to test each of these samples for poliomyelitis virus with negative results. Furthermore the strains recovered in newborn mice were tested in 8 additional monkeys and again the results were negative. The new virus was also found in the feces of 2 patients (No. 18-19) diagnosed as "fever of unknown origin". Lumbar puncture was done in only one of the latter patients and there was no pleocytosis.

The new virus has not been recovered by blind passage of brains of normal newborn mice, nor from separate fecal samples of 29 patients in New Haven with various other infectious and non-infectious diseases.

Fecal samples from two of the patients with non-paralytic disease (Nos. 11-12) yielded poliomyelitis virus when inoculated into monkeys, but did not produce disease in newborn mice. The 2 strains of poliomyelitis virus were typical; they were pathogenic for monkeys but not for cotton rats or mice (3 week old as well as newborn). Samples of feces from the 3 patients with clinical paralytic poliomyelitis (Nos. 13-15) failed to yield an agent when tested in either monkeys or newborn mice.

Tests for neutralization of the new virus with acute and convalescent sera of the patients revealed that:—(i) the sera of 7 patients (Nos. 1-5, 18, 19) from whom the new agent was isolated, neutralized the virus (neutralization index from 1,000 to 10,000) in the convalescent stage and to a lesser degree in the acute stage; (ii) the convalescent sera from 5 other patients (Nos. 6-10) with non-paralytic illnesses also neutralized the virus;

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<sup>†</sup> National Research Council Fellow in Medical Sciences.

<sup>1</sup> Dalldorf, G., and Sickles, G. M., *Science*, 1948, 108, 61.

TABLE I.  
Isolation of Virus and Appearance of Neutralizing Antibodies in Patients from Southern New England During August-October, 1948.

Patient*	Age	City	Diagnosis	Initial spinal fluid		Results of tests for virus		Neutralization of new virus by patient's convalescent serum
				WBC per cu mm	Ratio P : M	In monkeys (polio virus)	In newborn mice (new virus)	
1	9	New Haven	N.P.P.	31	33:67	—	+	+
2	5	"	A.M.	36	30:70	—	+	+
3	5	"	N.P.P.	27	7:93	—	+	+
4	11	Hartford	N.P.P.	125	54:46	—	+	+
5	32	"	N.P.P.	92	32:68	—	+	+
6	12	Providence	A.M.	600	15:85	—	—	+
7	6	New Haven	N.P.P.	129	25:75	—	—	+
8	18	Hartford	N.P.P.	140	74:26	—	—	+
9	6	New Haven	A.M.	42	47:53	N.T.	N.T.	+
10	12	Providence	N.P.P.	90	0:100	+	—	—
11	13	New Haven	A.M.	46	63:37	+	—	—
12	12	New Haven	N.P.P.	130	44:56	—	—	—
13	12	Stamford	P.P.	3	0:100	—	—	(INC.)
14	9	"	P.P.	43	70:30	—	—	—
15	13	Providence	P.P.	200	80:20	—	—	—
16	14	"	A.M.	142	49:51	—	—	—
17	15	Hartford	N.P.P.	95	—	—	—	—
18	14	New Haven	P.U.O.	3	0:100	N.T.	+	+
19	26	"	P.U.O.	N.T.	—	N.T.	+	+

P : M.—Ratio of Polymorphonuclear to Mononuclear Cells.

N.P.P.—Non-paralytic Poliomyelitis.

A.M.—Aseptic meningitis, cause unknown.

P.U.O.—Purpur of Unknown Origin.

P.P.—Paralytic Poliomyelitis.

N.T.—Not Tested.

INC.—Incomplete, acute sample 6 days after onset was negative, convalescent sample not tested.

\* We are indebted to the following physicians for their aid in obtaining specimens from these patients: Drs. Edward West and Alex A. Jaworski, Charles V. Chapin Hospital, Providence, R.I.; Dr. F. W. Burke, James W. McCook Memorial Hospital, Hartford, Connecticut; and Dr. Richard Shay, Englewood Hospital, Bridgeport, Connecticut.

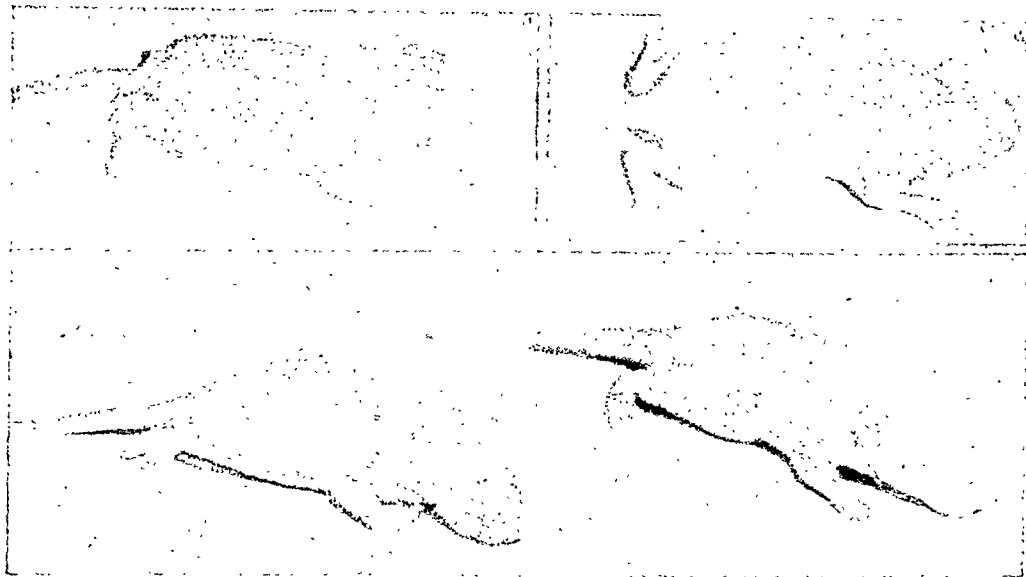


FIG. 1.

Two litter mates, the mouse on the left with paralysis of both legs and right arm (wrist-drop), the one on the right without signs of disease. Virus was inoculated intracerebrally and intraperitoneally on the 3rd day of life. Illness was first noted 6 days later when the photographs were taken.

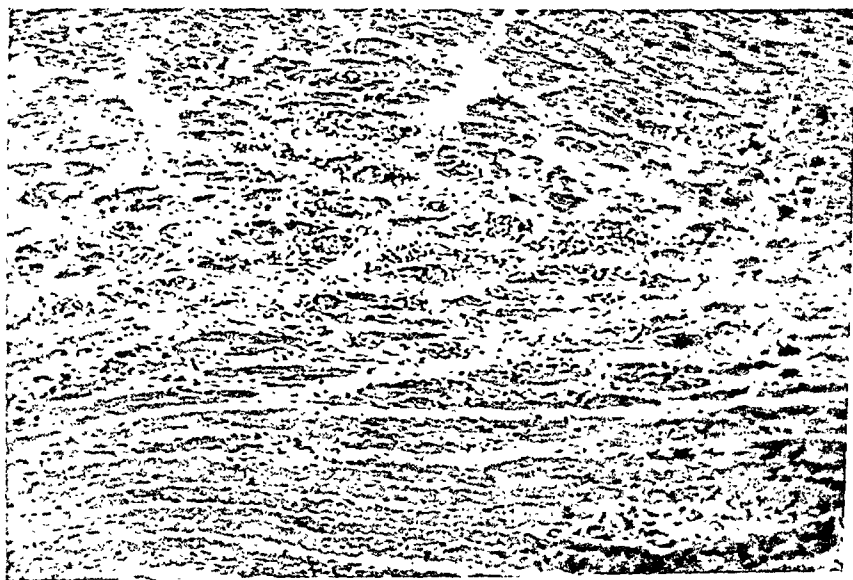


FIG. 2.

Myositis in paralyzed limb of 8-day-old mouse, sacrificed on the first day of paralysis. This animal was inoculated by the intracerebral and intraabdominal routes within 24 hours after birth. The photomicrograph was taken at 155 X magnification.

(iii) the convalescent sera of the 3 patients diagnosed as paralytic poliomyelitis and the serum of the 2 non-paralytic patients from whom poliomyelitis virus was isolated, all failed to neutralize the virus. The results of complement fixation tests with sera from these

patients indicated that mumps virus was not the etiologic agent of their illnesses.

(2) *Description of the experimental disease and some properties of the virus.* Swiss mice generally between the 1st and 2nd day of life have been used. Infectivity titers have ranged from  $10^{-5}$  to  $10^{-6}$  with suspensions of brain and slightly higher with suspensions of muscle. The mice have been inoculated intracerebrally and/or intraperitoneally. Signs of disease have appeared within 2 to 10 days, manifested by weakness and paralysis of one or more extremities (Fig. 1) and followed by death generally within 24 hours. In very young mice marked ataxia may be the only sign before death. The outstanding pathological finding has been an extensive myositis in the skeletal muscles especially of the limbs (Fig. 2). In some mice lesions have been noted in the heart muscle and in the brain.

Aerobic and anaerobic cultures of suspensions of tissues used for passage have shown no bacterial growth on ordinary media. The agent as it occurs in human feces and in mouse brain used for passage, has been passed through a tested, Corning, bacterial, glass-fritted filter. The agent is resistant to the action of ether, penicillin, streptomycin and chloromycetin. It has failed to produce disease in 3 week old mice, cotton rats, snowball rats, albino rats, hamsters as well as in young adult monkeys.

Studies on the distribution of the virus in newborn mice indicate that it is widespread at the time of paralysis; it has been recovered from the blood, brain, muscles, heart, liver, spleen and also from the intestinal contents. That the agent recovered from this latter source was not Theiler's TO virus was indicated by its lack of pathogenicity for 3 week old mice.

Preliminary data on sedimentation suggest that the agent is one of the smaller viruses. Thus, although some of the virus may be thrown down together with the particles which sediment at 18,000 r.p.m. for 30 minutes (6 inch rotor), most of the virus remains in the supernatant fluid. It may be sedimented readily at 36,000 r.p.m. for 60 minutes.

Neutralization tests have been carried out

in newborn mice by the intraperitoneal route. It was found that the virus is readily neutralized by homologous antiserum prepared by repeated inoculation of mice or monkeys. It is not neutralized by antiserum from animals hyperimmunized against the following viruses; poliomyelitis (Lansing strain, North Carolina 1948 strain, Texas 1948 strain), Theiler's FA and GDVII strains of mouse encephalomyelitis virus, mumps, herpes, lymphocytic choriomeningitis, encephalomyocarditis, louping ill, Venezuelan equine encephalitis, and Newcastle disease.

(3) *Infection of chimpanzees.* As mentioned above, rhesus monkeys do not appear to be susceptible to infection with this virus when inoculated intracerebrally, intraperitoneally, intramuscularly, intracutaneously or subcutaneously. Two chimpanzees were each given one oral administration of the virus. No clinical signs of illness developed. However, virus was recovered from their feces for 12 days after the feeding and from their throats on the 5th, 6th and 8th days. Neutralizing antibodies, absent before exposure to virus, were present on the 14th and 28th days.

(4) *Accidental infection of man.* One of the physicians engaged in work with this agent developed a vague febrile illness of 8 days duration which was diagnosed as "fever of unknown origin." The only suggestion of involvement of the central nervous system was minimal stiffness of the back; a spinal tap was not done. Virus was recovered from the feces and nasopharyngeal washings during the acute illness. Neutralizing antibodies were not found in serum collected before or during the early acute phase of illness, but appeared in increasing titer during convalescence. The neutralization index on the 43rd day after onset of illness was 10,000.

(5) *Occurrence of the virus in "poliomyelitis" patients in Ohio, 1947 and North Carolina, 1948.* Samples of feces collected from different parts of the country and frozen since collection were also examined for the virus. It was not found in pooled specimens collected during 1944 from patients with paralytic poliomyelitis in New York City, nor from similar specimens collected from patients in

Los Angeles, California in 1948: These pooled samples were proven by monkey inoculation to contain poliomyelitis virus. The new virus, as well as poliomyelitis virus, was isolated from pooled fecal samples of 6 patients who had non-paralytic poliomyelitis in 1947 in Akron, Ohio. Both viruses were also present in pooled fecal samples collected in 1948 from patients with paralytic poliomyelitis in Winston-Salem, N. C. An ultracentrifuged concentrate of the Winston-Salem specimens had titers of  $10^{-3}$  for poliomyelitis virus in monkeys and of  $10^{-3.5}$  for the new virus in newborn mice.

(6) *Occurrence of the virus in extra-human sources.* The virus has been sought in samples of the sewage from 6 cities, 3 situated in Connecticut (Hartford, Norwalk, New Haven) and 3 in North Carolina (Greensboro, High Point, and Winston-Salem), and in non-biting flies trapped in 2 of these cities. In the summer of 1948 "mild poliomyelitis" appeared to be prevalent in Connecticut, and a severe epidemic of classical poliomyelitis occurred in North Carolina. As already mentioned, the new virus was isolated from patients in both areas.

The new virus was found in the sewage of all of the above 6 cities during some part of the summer and fall of 1948 and usually several serial samples from each city were positive. Serial sampling during the winter has for the most part been negative but the data are too few to indicate whether the occurrence of the virus in sewage follows the seasonal pattern of poliomyelitis virus.<sup>2,3</sup>

Tests of flies from the above areas for the presence of the new virus have not yet been completed, but thus far 2 isolations have been obtained, one from flies trapped in August, 1948 in Hartford, Connecticut and another from flies trapped in July, 1948 in High Point, N. C. In addition to studying this material from Connecticut and North Carolina, flies from Texas were also examined. These were trapped serially in 1948 during an epidemic of poliomyelitis in the lower Rio Grande

Valley.<sup>4</sup> From some batches of flies, separated according to species, both the new virus and poliomyelitis virus have been isolated. Other batches of flies have yielded either one virus or the other, and many batches have yielded neither virus. The species of flies which have given positive tests for both viruses are (a) *Musca domestica*, (b) *Phaenicia sericata* and *P. pallescens*, and (c) *Sarcophagula* and *Sarcophaga* spp. The two viruses (poliomyelitis and the new virus isolated in mice) even when obtained from the same batch of flies do not appear to be related by the tests we have thus far used (host range, virus neutralization by hyperimmune sera.)

(7) *Immunological types.* By means of cross neutralization tests, it has been found that two strains of the murine infecting virus isolated from patients in New Haven are related to each other as well as to a strain isolated from Hartford sewage. Furthermore a strain from Texas flies was found to be related to a North Carolina sewage strain, but not to the Connecticut strains. Thus each strain was readily neutralized by homologous hyperimmune sera, but antisera against Connecticut strains failed to neutralize the Texas virus, and, in similar fashion, Texas antisera had no effect on the Connecticut virus.

*Summary.* This paper reports the isolation of a filtrable virus from the feces of patients diagnosed either as non-paralytic poliomyelitis or aseptic meningitis and from 2 patients with "fever of unknown origin." The agent is similar to that reported by Dalldorf and Sickles<sup>1</sup> in producing paralysis with myositis in newborn mice. The recovery of virus was correlated with the appearance of neutralizing antibodies in the patients' sera. At least two immunological types of the virus exist. The virus was widespread in this country during the summer of 1948 having also been isolated from the sewage of a number of

<sup>2</sup> Trask, J. D., and Paul, J. R., *J. Exp. Med.*, 1942, 75, 1.

<sup>3</sup> Melnick, J. L., *Am. J. Hygiene*, 1947, 45, 240.

<sup>4</sup> The flies in Texas were caught and identified by Dr. Richard P. Dow, Entomologist, and Staff, attached to the Dysentery Control Project, U. S. Public Health Service. They were collected as a part of field studies on epidemic poliomyelitis being carried out by this unit.

cities and from flies collected in widely separated areas. Subclinical infection may be produced in chimpanzees by oral adminis-

tration of the virus. A laboratory worker has been accidentally infected with the virus.

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# 17187. Effect of Phenergan (N-Dimethylamino-2-Propyl-1-Thiodiphenylamine, 3277 RP) on the Arthus Reaction in Rabbits.

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The so-called antihistaminic compounds have been of great value in that they have made possible the study of various biological phenomena, principally those related to histamine reactions, and the treatment of histamine-like allergies. As yet, none of the antihistaminics have been found to be effective in modifying the necrotizing type of allergic reactions. The lack of effect of pyribenzamine on the Arthus phenomenon and bacterial allergic reactions has been reported.<sup>1</sup> The present study is presented because an unusually potent antihistaminic, possessing, in addition, properties apparently unrelated to its antagonism to histamine, has been found effective in inhibiting the Arthus reaction.

The Fourneau compounds<sup>2,4</sup> and antergan<sup>2</sup> were modified to produce phenergan, N-dimethylamino - 2 propyl - 1-thiodiphenylamine, (3277 RP) which was extensively studied by Halpern.<sup>5,6,7</sup> In addition to being

30 times more effective than antergan in protecting guinea pigs against histamine, phenergan apparently protects capillaries against injury by various noxious stimuli.<sup>8-12</sup> The drug was kindly given for this study by Dr. B. N. Halpern. A quantitative technic for the induction of the Arthus reaction with known quantities of a single antigen and its homologous antibody has been described.<sup>1,13</sup> This method was employed in the present study.

*Experimental.* Arthus reactions were simultaneously induced in untreated control rabbits and in those treated with varying amounts of phenergan (25 to 100 mg/kg). In preliminary experiments, the Arthus reaction was induced in multiple sites in 9 albino rabbits by the local injection of antibody and of antigen as previously described.<sup>1</sup> It was found that 75 mg kilo of phenergan in saline intramuscularly prevented the development of a severe Arthus reaction. The technic for producing the Arthus reaction was then modified by the administration of the antigen intraven-

\* Work done during the tenure of a Life Insurance Medical Research Fellowship.

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<sup>1</sup> Fischel, E. E., *Proc. Soc. Exp. Biol. and Med.*, 1947, **66**, 537.

<sup>2</sup> Halpern, B. N., *Comp. r. Soc. de Biol.*, 1946, **140**, 361.

<sup>3</sup> Fourneau, E., and Boret, D., *Arch. Intern. Pharmacol. et Ther.*, 1933, **46**, 178.

<sup>4</sup> Staub, A. M., *Ann. Inst. Pasteur*, 1939, **63**, 400, 485.

<sup>5</sup> Halpern, B. N., *Arch. Intern. Pharmacol. et Ther.*, 1947, **74**, 314.

<sup>6</sup> Halpern, B. N., *J. Allergy*, 1947, **18**, 263.

<sup>7</sup> Halpern, B. N., *Acta Allergologica*, 1948, **1**, 3.

<sup>8</sup> Halpern, B. N., and Cruehaud, S., *Comp. r. Soc. de Biol.*, 1947, **141**, 1038.

<sup>9</sup> Halpern, B. N., Hamburger, F., and Cruehaud, S., *Acta Allergologica*, 1948, **1**, 97.

<sup>10</sup> Halpern, B. N., and Cruehaud, S., *Comp. r. Acad. des Sciences*, 1947, **225**, 1194.

<sup>11</sup> Hamburger, J., Halpern, B. N., and Neel, Z., *Comp. r. Soc. de Biol.*, 1948, **142**, 153.

<sup>12</sup> Halpern, B. N., personal communication.

<sup>13</sup> Fischel, E. E., and Kaba, E. A., *J. Immunol.*, 1947, **55**, 337.

TABLE I.

Effect of Phenergan on Severity of the Reverse Arthus Reaction Induced Passively in Animals Injected Intravenously with 1.5 to 2.0 mg of Crystalline Egg Albumin Nitrogen, and 30 Minutes Later Injected Intradermally with Known Amounts of Anti-Egg Albumin.  
Number of reactions of different severity related to total reactions in each group.

Severity of the reaction	Phenergan 100 mg/kg	Phenergan 75 mg/kg	Phenergan 50 mg/kg	Phenergan 25 mg/kg	Control animals
0.22 mg AbN.					
0	0	0	0	0	0
±	0	2/26	0	0	0
+	1/20	20/26	3/20	0	4/38
++	18/20	4/26	13/20	4/4	5/38
+++	0	0	4/20	0	3/38
++++	1/20	0	0	0	26/38
0.025 mg AbN.					
0	1/4	2/18	0	0	0
±	3/4	8/18	4/4	2/4	5/14
+	0	8/18	0	2/4	8/14
++	0	0	0	0	1/14

ously and the subsequent injection of antibody intracutaneously.<sup>13</sup> This procedure was found to result in a more rapidly developing and more hemorrhagic reaction than did the local administration of both antigen and antibody. The antigen was administered intravenously to 45 rabbits in amounts of 1.5 to 2.0 mg egg albumin nitrogen (EaN); 30 minutes later the antibody was injected intracutaneously in amounts of 0.22 and 0.025 mg antibody nitrogen (AbN). Untreated controls were injected simultaneously with phenergan treated animals. The treated group of animals received 2 doses of phenergan in amounts of 25, 50, 75 or 100 mg/kg intramuscularly. The first dose was given at the time the antigen was administered; and the second dose, 4 hours later. One of 6 animals given 100 mg/kg phenergan had convulsions after the second dose and died. The other animals showed some drowsiness, muscular incoordination and occasional muscular twitchings with the dosages of 75 or 100 mg/kg but recovered completely in a few hours. The skin reactions were observed at 6 and 24 hours. In this series of rabbits the most acutely severe reactions appeared at 6 hours after the injections of the antibody. The reactions were graded as follows:

0 = no reaction; + = petechial hemorrhages of an area less than 1 cm in diameter with minimal edema; ++ = less than 1 cm of confluent intracutaneous hemorrhage or 1 cm of petechial hemorrhage with edema over

a somewhat larger area (2 cm); +++ = 1 cm of confluent hemorrhage or 2 cm of petechial hemorrhage with moderate edema; ++++ = 1.5 cm or more of confluent hemorrhage, with moderate to severe edema. The results are presented in Table I.

In Table I, it is apparent that phenergan has a definite inhibitory effect on the severity of the reaction at the higher concentrations of antibody (0.22 mg AbN). When only 0.025 mg AbN was employed, there did not appear to be any significant difference between the control animals and those receiving phenergan. It is much more difficult to compare minimally severe reactions employing the criteria used in this study. In consideration of the sedative and anesthetic side effects of phenergan, two animals were given respectively procaine and pentobarbital intramuscularly and were given both antigen and antibody locally. The severity of the reactions in these animals, although not tabulated, was similar to that of untreated control animals. In Table I, with the maximum amount of antibody (0.22 mg AbN), 29 out of 38 reactions, or 75%, in the control group were graded +++ or more. In the treated group, only 5 of 70 reactions, or 7%, were similarly graded. Since the dose of phenergan administered here is far greater than the dose of pyribenzamine used in the previous study (10 mg/kg),<sup>1</sup> it seemed relevant to determine whether pyribenzamine given in comparable amounts would affect the Arthus reaction.

Two rabbits were given 25 mg/kg and 75 mg/kg respectively of pyribenzamine intramuscularly in a single dose and died shortly thereafter with severe convulsions. To investigate further the activity of phenergan, the effect of the drug was studied on the skin reaction of the rabbit to the streptococcal erythrogenic toxin. Two control animals observed 24 hours after the injection of 2 skin sites with 550 skin test doses of toxin showed areas of edema and erythema 1.5 cm in diameter. Milder edema and erythema resulted after the injection of 55 skin test doses into two other sites. In contrast, 2 other animals treated with 75 mg/kg of phenergan every 4 hours for 3 doses showed no reaction to either the 55 or the 550 skin test doses of erythrogenic toxin. The observation that phenergan inhibits the effect of the erythrogenic toxin is consistent with the view that the drug appears to protect the capillary bed from injury.

*Discussion.* The severity of the Arthus reaction can be related to the amount of precipitin available for union with antigen locally.<sup>13,14</sup> The primary site of the injury due to the antigen-antibody reaction is probably the small blood vessels and capillaries.<sup>15</sup> Certain observations indicate that the action of phenergan is on these small blood vessels. Halpern and his associates have shown that the drug, in doses of 20 mg/kg, can protect rabbits against pulmonary edema induced by the injection of such unrelated agents as epinephrine and chloropicrin, a war gas.<sup>8-10</sup> Furthermore, Hamburger *et al.*<sup>11</sup> have studied the effect of phenergan on experimental orthostatic albuminuria and hematuria in rabbits,

and found that the animals were completely protected by doses of 20 mg/kg. The inhibition of the Schwartzman reaction in rabbits has been noted recently.<sup>12</sup> These observations suggest that the effect of the drug on the Arthus reaction may well be a consequence of its ability to decrease capillary damage in the presence of nonspecific irritants. As such, the mechanism of action would be on the vascular bed and have little relation to the occurrence of an antigen-antibody reaction.

The dosages of phenergan employed in this study (25 to 100 mg/kg of body weight) were not lethal for the rabbit except for the highest dose, 100 mg/kg, which caused death in 1 of 6 rabbits. These doses are far greater than the amounts used therapeutically in humans, and, indeed, are the highest reported in animals. Halpern and Hamburger used 20 mg/kg effectively against experimental pulmonary edema and orthostatic albuminuria. The dose of phenergan which is effective as an antihistaminic is still smaller.

*Summary.* The effect of phenergan on the Arthus reaction was investigated, employing a quantitative method for the induction of the reaction. In large but non-lethal doses, the drug was found to inhibit appreciably the development of the more severe Arthus reactions. It is suggested that the action of the drug on the Arthus reaction is related to its effect on capillary permeability. A limited study of the inhibition by phenergan of the toxic effect of a large quantity of streptococcal erythrogenic toxin appears to support this view.

We wish to thank Dr. Elvin A. Kabat for his encouragement and helpful criticism.

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<sup>14</sup> Opie, E. L., *J. Immunol.*, 1924, 9, 231.

<sup>15</sup> Abell, R. G., and Schenk, H. P., *J. Immunol.*, 1938, 34, 195.



TABLE I.

Effect of Phenergan on Severity of the Reverse Arthus Reaction Induced Passively in Animals Injected Intravenously with 1.5 to 2.0 mg of Crystalline Egg Albumin Nitrogen, and 30 Minutes Later Injected Intradermally with Known Amounts of Anti-Egg Albumin.  
Number of reactions of different severity related to total reactions in each group.

Severity of the reaction	Phenergan 100 mg/kg	Phenergan 75 mg/kg	Phenergan 50 mg/kg	Phenergan 25 mg/kg	Control animals
0.22 mg AbN.					
0	0	0	0	0	0
±	0	2/26	0	0	0
+	1/20	20/26	3/20	0	4/38
++	18/20	4/26	13/20	4/4	5/38
+++	0	0	4/20	0	3/38
++++	1/20	0	0	0	26/38
0.025 mg AbN.					
0	1/4	2/18	0	0	0
±	3/4	8/18	4/4	2/4	5/14
+	0	8/18	0	2/4	8/14
++	0	0	0	0	1/14

ously and the subsequent injection of antibody intracutaneously.<sup>13</sup> This procedure was found to result in a more rapidly developing and more hemorrhagic reaction than did the local administration of both antigen and antibody. The antigen was administered intravenously to 45 rabbits in amounts of 1.5 to 2.0 mg egg albumin nitrogen (EaN); 30 minutes later the antibody was injected intracutaneously in amounts of 0.22 and 0.025 mg antibody nitrogen (AbN). Untreated controls were injected simultaneously with phenergan treated animals. The treated group of animals received 2 doses of phenergan in amounts of 25, 50, 75 or 100 mg/kg intramuscularly. The first dose was given at the time the antigen was administered; and the second dose, 4 hours later. One of 6 animals given 100 mg/kg phenergan had convulsions after the second dose and died. The other animals showed some drowsiness, muscular incoordination and occasional muscular twitchings with the dosages of 75 or 100 mg/kg but recovered completely in a few hours. The skin reactions were observed at 6 and 24 hours. In this series of rabbits the most acutely severe reactions appeared at 6 hours after the injections of the antibody. The reactions were graded as follows:

0 = no reaction; + = petechial hemorrhages of an area less than 1 cm in diameter with minimal edema; ++ = less than 1 cm of confluent intracutaneous hemorrhage or 1 cm of petechial hemorrhage with edema over

a somewhat larger area (2 cm); +++ = 1 cm of confluent hemorrhage or 2 cm of petechial hemorrhage with moderate edema; ++++ = 1.5 cm or more of confluent hemorrhage, with moderate to severe edema. The results are presented in Table I.

In Table I, it is apparent that phenergan has a definite inhibitory effect on the severity of the reaction at the higher concentrations of antibody (0.22 mg AbN). When only 0.025 mg AbN was employed, there did not appear to be any significant difference between the control animals and those receiving phenergan. It is much more difficult to compare minimally severe reactions employing the criteria used in this study. In consideration of the sedative and anesthetic side effects of phenergan, two animals were given respectively procaine and pentobarbital intramuscularly and were given both antigen and antibody locally. The severity of the reactions in these animals, although not tabulated, was similar to that of untreated control animals. In Table I, with the maximum amount of antibody (0.22 mg AbN), 29 out of 38 reactions, or 75%, in the control group were graded +++ or more. In the treated group, only 5 of 70 reactions, or 7%, were similarly graded. Since the dose of phenergan administered here is far greater than the dose of pyribenzamine used in the previous study (10 mg/kg),<sup>1</sup> it seemed relevant to determine whether pyribenzamine given in comparable amounts would affect the Arthus reaction.

TABLE I.  
Effect of Isuprel upon Level of Blood Glucose at the End of 2 Hours in Eviscerate Rats Given Glucose and Insulin by Constant Injection.

Exp.	Conc. isuprel	No. rats	Avg blood glucose	Avg*	Diff. in avg	Diff.*	Diff. diff.*
1	1:25,000 0	12	161	7.9			
		12	84	6.9	77	10.5	7.3
2	1:10,000 0	12	185	9.1			
		12	84	5.2	101	10.5	9.6

\* Standard deviation.

of isuprel in concentrations of 1:10,000 and 1:25,000 caused a marked decrease in the glucose tolerance of the eviscerate rats.

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### 17189. Effect of Testosterone Propionate on Phosphatases in the Seminal Vesicle and Prostate of the Rat.\*

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The discovery by Kutscher and Wolbergs<sup>1</sup> that the human prostate gland and ejaculum contain high concentrations of acid phosphatase provided the stimulus for a series of investigations of this enzyme in the tissues and secretions of the male reproductive tract.

Gutman and Gutman<sup>2</sup> showed that the high level of acid phosphatase (AcP-ase) as well as alkaline phosphatase (Alp-ase) in the prostatic tissue of the adult rhesus monkey is not present in the prepubertal animal, but that these enzymes can be raised to the adult level by the injection of androgen.

Pazos and Huggins<sup>3</sup> found that administration of androgen to starving, castrate im-

mature dogs caused an 8 to 11-fold increase in AcP-ase activity of the prostate gland over that of a control animal. The Alp-ase in the glands of these dogs decreased 15-45%.

When Gutman and Gutman<sup>4</sup> studied the rat, however, they found that the levels of both AcP-ase and Alp-ase in the prostate gland were extremely low compared to levels in the primates studied. Administration of testosterone to normal adult male rats produced no change in the activity of either enzyme.

Atkinson<sup>5</sup> has shown histochemically that the Alp-ase in the stromal elements of the mouse seminal vesicle diminishes after castration and returns to normal after androgen treatment. Dempsey, Greep, and Deane<sup>6</sup> recently demonstrated the same phenomenon in the rat, also by histochemical means.

It was decided to conduct investigations to (1) determine whether an enzymatic response to testosterone could be obtained in the

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<sup>†</sup> National Cancer Institute Predoctorate Research Fellow, National Institute of Health.

<sup>1</sup> Kutscher, W., and Wolbergs, H., *Z. physiol. Chem.*, 1935, 230, 237.

<sup>2</sup> Gutman, A. B., and Gutman, E. B., *Proc. Soc. Exp. Biol. and Med.*, 1939, 41, 277.

<sup>3</sup> Pazos, R., and Huggins, C., *Endocrinology*, 1945, 30, 416.

<sup>4</sup> Gutman, A. B., and Gutman, E. B., *Proc. Soc. Exp. Biol. and Med.*, 1938, 39, 529.

<sup>5</sup> Atkinson, W. B., *Anat. Rec.*, 1948, 100, 731.

<sup>6</sup> Dempsey, E. W., Greep, R. O., and Deane, H. W., *Endocrinology*, 1949, 44, 88.

## 17188. Effect of Isuprel Upon Tolerance of the Eviscerate Rat for Glucose.

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Epinephrine<sup>1</sup> and posterior pituitary extracts<sup>2</sup> have similar effects upon the tolerance of the eviscerate rat for glucose. When a solution of glucose without insulin is administered to the eviscerate rat, the addition of epinephrine or posterior pituitary extract to the solution has little or no effect upon the level of blood glucose during the first 2 hours but over a period of 24 hours there is an increase in glucose tolerance. When insulin is given with glucose, the addition of either epinephrine or posterior pituitary extract caused a rapid decrease in glucose tolerance.

It was proposed to extend these studies by testing a derivative of epinephrine having a depressor action upon blood pressure. In the present study, isuprel (isopropylepinephrine) was found to behave as epinephrine and the pressor principle of the posterior pituitary in causing a striking decrease in the glucose tolerance of the eviscerate rat given glucose and insulin.

*Methods.* The two-stage procedure of evisceration has been described.<sup>3</sup> When male rats of the Sprague-Dawley strain reached  $250 \pm 2$  g, they were anesthetized (cyclopal) and subjected to the second stage of evisceration. Intravenous infusions of solutions containing glucose (C.P. Dextrose, Merck) and insulin (Regular Insulin, Lilly) were made by a continuous injection machine which delivered fluid into the saphenous vein of the right hind leg at the rate of 20 cc per 24 hours for each of 6 rats simultaneously. The glucose load was 64 mg per 100 g of rat per hour and the insulin dose was 4 units per 24 hours per rat. The temperature was constant at  $26.5 \pm 0.5^\circ\text{C}$ . At the end of 2 hours,

jugular vein blood was analyzed for glucose by the method of Miller and Van Slyke.<sup>4</sup>

*Experiments and results.* In Experiment 1, isuprel was administered to one rat of each of 12 pairs by adding it to the infusion fluid in a concentration of 1:25,000. In Exp. 2, isuprel was administered to one rat of each of 12 pairs by adding it to the infusion fluid in the concentration of 1:10,000. As shown in Table I, the addition of isuprel caused a striking decrease in glucose tolerance in both experiments.

*Discussion.* The effects of isuprel upon blood pressure were not studied under these experimental conditions. Although this compound has been described<sup>5</sup> as having a vaso-depressor action in experimental animals, it would be unsafe to assume that its vasomotor effects are dissimilar from those of epinephrine and posterior pituitary extracts in the eviscerate rat. It was noted that isuprel caused the skin to become pink, suggesting peripheral vasodilatation, in contrast to the pallor caused by the peripheral vasoconstriction which occurs during the administration of epinephrine or of posterior pituitary extract under identical conditions. The mechanism whereby these drugs affect glucose tolerance in the eviscerate rat is not known but the possibility that it is secondary to circulatory changes must be considered.

*Summary.* Eviscerate rats (250 g) were given continuous intravenous infusions of glucose and insulin during a period of 2 hours. The glucose load was 64 mg of glucose per 100 g of rat per hour and insulin was given at the rate of 4 units per rat per 24 hours. The solution of glucose and insulin was infused at the rate of 20 cc per 24 hours. The addition

<sup>1</sup> Ingle, D. J., and Nezamis, J. E., *Am. J. Physiol.*, 1949, 156.

<sup>2</sup> Ingle, D. J., and Nezamis, J. E., *Am. J. Physiol.*, 1949, 156.

<sup>3</sup> Ingle, D. J., *Exp. Med. and Surg.*, 1949, 7, 34.

<sup>4</sup> Miller, B. F., and Van Slyke, D. D., *J. Biol. Chem.*, 1936, 114, 583.

<sup>5</sup> Lands, A. M., Nash, V. L., Dertinger, B. L., Granger, H. R., and McCarthy, H. M., *J. Pharm. and Exp. Therap.*, 1948, 92, 369.

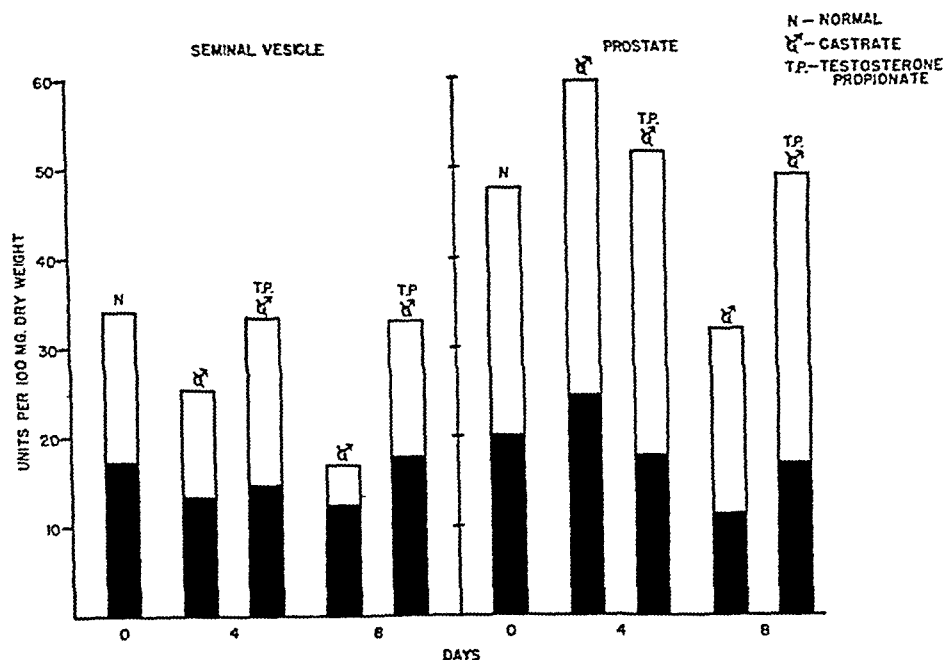


FIG. 1.

Changes in phosphatase activity produced by castration and treatment with testosterone propionate. AIP-ase activity is represented by total height of the bars. AcP-ase activity is represented by the black portions of the bars.

In the castrate animals receiving testosterone propionate, the androgen largely prevents the castration changes in weight and enzyme activity. The AIP-ase is maintained at almost exactly the same level in the androgen-treated animals as in the normals, while the AcP-ase activity is fully restored to the normal level by the eighth day of treatment. These data are presented in Table I and Fig. 1. The results clearly show that deprivation of androgen results in a loss of activity of both phosphatases in the seminal vesicle and that replacement therapy with testosterone propionate restores the activity.

**Prostate Gland.** The enzyme pattern in the prostate is similar to that obtained in the seminal vesicle with one major difference. While the castration effects are manifested in the seminal vesicle by the fourth day after castration, in the prostate the effects are not clearly shown until the eighth day. There is greater variability in the prostate values, probably caused by the variation in amount of secretion present in the tissues, as the technic for removal of the secretion may not

be completely effective.

By the fourth day after castration, 2 out of 3 animals did not show significant losses of weight in the ventral lobe of the prostate, while the AcP-ase and AIP-ase actually increased in activity by 20 and 25% respectively. By the eighth day, however, the weight of the gland was about one-third of the normal weight and the AcP-ase and AIP-ase activities were reduced by one-half and one-third respectively.

When testosterone propionate was administered to the castrate animals, the AIP-ase of the gland was restored to normal and the AcP-ase activity was partially restored. The dose of testosterone propionate used was sufficient to cause great prostatic hypertrophy by the eighth day. These data are presented in Table II and in Fig. 1.

Histochemical treatment (Gomori<sup>2</sup>) of the prostate to demonstrate localization of the AIP-ase revealed a possible reason why

<sup>2</sup> Gomori, G., *Proc. Soc. Exp. Biol. and Med.*, 1939, 42, 23.

TABLE I.  
Changes in Seminal Vesicle Following Castration and Treatment with Testosterone Propionate.

Experimental stage	No. of rats*	Wt of one seminal vesicle (mg)	AcP-aset	AlP-aset	Dry wt (%)
Normal	4	160 (150-167)	17 (16-18)	34 (22-46)	20
Castrate 4 days	3	120 (112-130)	13 (11-15)	25 (20-29)	19
" 8 "	3	80 (77-86)	12 (11-14)	17 (13-20)	22
Testosterone 4 days	3	375 (227-522)	14 (12-17)	33 (31-34)	20
" 8 "	3	275 (273-279)	18 (14-22)	33 (28-37)	21

\* Pertains to enzyme values only. Gland weights and dry weights taken from two rats in each group.

† Expressed as Huggins-Talalay units per 100 mg dry tissue. Figures in parentheses indicate ranges.

rat prostate similar to that obtained in the monkey and in the dog, (2) to provide quantitative support for the histochemical findings on the AlP-ase of the seminal vesicle, and (3) to provide information on the hitherto uninvestigated AcP-ase in the seminal vesicle. An experiment was designed, therefore, to study both AcP-ase and AlP-ase in homogenates of prostates and seminal vesicles of normal rats, castrate rats and in castrate rats receiving testosterone propionate.

*Methods.* The animals used were male rats of the Sprague-Dawley strain, 2½ to 3 months old. The rats were divided into 3 groups: (1) Normal animals, untreated; (2) Castates, untreated; (3) Castates injected subcutaneously with 600  $\gamma$  testosterone propionate per day in 2 daily doses. Animals of groups (2) and (3) were killed 4 and 8 days following castration.

At autopsy the ventral lobe of the prostate was removed and weighed. The tissue was laid on moist, hard-surfaced filter paper and minced with a scalpel blade to rupture the majority of the gland lobules and permit escape of the contained secretion. The chopped tissue was washed in cold glass-distilled water, blotted on filter paper, weighed, and transferred to a chilled glass homogenizing tube. One seminal vesicle was removed and separated from the adherent coagulating gland; the secretion was expressed, and the gland was washed with cold saline solution, blotted, and transferred to an homogenizing tube.

Tissues were homogenized in glass distilled water, one per cent homogenates being used throughout the study. Solids and water con-

tent of the tissues were determined by drying pieces of tissue or homogenates at 75°C for 24 hours.

The Huggins-Talalay method<sup>7</sup> of phosphatase estimation, modified as previously reported,<sup>8</sup> was used on all tissues. This method measures phenolphthalein liberated from phenolphthalein phosphate. Both AcP-ase and AlP-ase were determined on tissues from at least three animals in all stages studied. The sequence in which the experiments were made was randomized in order to distribute any variation caused by minor differences in technic and condition of the animals due to seasonal factors.

*Results. Seminal Vesicle.* After castration the seminal vesicle rapidly loses weight, undergoing a 25% reduction in 4 days and reaching a level 50% below the pre-castration level by 8 days. The AlP-ase activity is reduced 30% in the first 4 days after castration, reaching a level 50% below the normal by the eighth day. The proportionality between weight loss of the gland and loss of enzyme activity is striking. Enzyme activity is expressed in terms of activity per unit weight of dry tissue, however, and the reduction in activity is real, not merely a reflection of the reduction in size of the gland.

The AcP-ase follows a trend slightly different from the gland weight and the AlP-ase, being reduced in activity by 25% after 4 days of castration, then undergoing very little further reduction through the eighth day.

<sup>7</sup> Huggins, C., and Talalay, P., *J. Biol. Chem.*, 1945, 159, 399.

<sup>8</sup> Stafford, R. O., McShan, W. H., and Meyer, R. K., *Endocrinology*, 1947, 41, 45.

tially normal levels by androgen therapy. The seminal vesicle is more sensitive than the prostate gland in this respect.

Androgen therapy caused hypertrophy of the glands beyond normal size, but under the

conditions of these experiments, the acid and alkaline phosphatase activity was not increased beyond normal levels.

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### 17190. Investigations on the Use of Eserine for the Differentiation of Mammalian Esterases.\*

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In most mammalian tissues there exist 3 types of esterase<sup>1</sup> capable of facilitating the hydrolysis of noncholine aliphatic esters, *i.e.*, ali-esterase,<sup>2</sup> pseudo-cholinesterase<sup>1</sup> (s-type)<sup>3</sup> and true cholinesterase<sup>1</sup> (e-type).<sup>3</sup> Most esters of aliphatic acids can be hydrolysed by enzymes from two of these 3 groups, certain non-choline esters (*e.g.*, triacetin) seem to be hydrolysed by enzymes of all 3 types<sup>4-6</sup> and, further, esterases of all 3 types are present in most crude tissue preparations. To distinguish between that portion of the hydrolysis of aliphatic esters which is due to the cholinesterases and that portion which is due to the ali-esterases in tissue preparations, Easson and Stedman<sup>7</sup> employed low concentrations of eserine or prostigmine to inhibit selectively any activity due to the cholinesterases (ChE's). Later investigators, *e.g.*, Richter and Croft,<sup>2</sup> Mendel and co-workers,<sup>1</sup> and others, also found low concentrations of

eserine to be a very satisfactory tool for differentiating cholinesterase and ali-esterase activity. Thus it has been found that a concentration of  $10^{-6}$ M eserine inhibits completely, or almost completely, the activities of both the true and the pseudo-ChE's of most mammalian tissues while the activity of the ali-esterases remains relatively unaffected by as much as 100 times this concentration.

Recently, however, McNaughton and Zeller<sup>8</sup> have concluded from their observations that "the use of eserine to decide whether a hydrolysis is catalysed by a cholinesterase or another esterase is of limited value because the s-type (pseudo-cholinesterase) is inhibited by this substance only in the presence of acetylcholine but not in that of ethyl chloroacetate." The evidence obtained in the present investigation has indicated, however, that eserine in low concentrations ( $10^{-6}$ M) can, in fact, be used to inhibit completely the activity of pseudo-ChE toward ethyl chloroacetate (ECIA) and that differentiation of cholinesterase activity from ali-esterase activity is therefore still possible by this means. Moreover, even though this concentration of eserine may not be adequate to effect complete inhibition of the ChE's from certain non-mammalian organisms,<sup>9</sup> in no case tested has the sensitivity of any cholinesterase to eserine been found to be altered significantly when one

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\* Supported by a grant from the National Research Council of Canada.

<sup>1</sup> Mendel, B., and Rudney, H., *Biochem. J.*, 1943, 37, 59.

<sup>2</sup> Richter, D., and Croft, P. G., *Biochem. J.*, 1942, 30, 746.

<sup>3</sup> Zeller, E. A., and Bissegger, A., *Helv. chim. acta*, 1943, 26, 1619.

<sup>4</sup> Bodansky, O., *Ann. N. Y. Acad. Sci.*, 1946, 47, 521.

<sup>5</sup> Hawkins, R., Ph.D. Thesis, Toronto, 1947.

<sup>6</sup> Adams, D. H., and Whittaker, V. P., *Biochem. J.*, 1948, 43, xiv.

<sup>7</sup> Easson, E. H., and Stedman, E., *Biochem. J.*, 1937, 31, 1723.

<sup>8</sup> McNaughton, R. A., and Zeller, E. A., *Proc. Soc. Exp. Biol. and Med.*, 1949, 70, 165.

<sup>9</sup> Hawkins, R. D., and Mendel, B., *J. Cell. Comp. Physiol.*, 1946, 27, 69.

TABLE II.

Changes in Prostate Gland Following Castration and Treatment with Testosterone Propionate.

Experimental stage	No. of rats*	Wt of ventral prostate (mg)	AcP-ase†	AlP-ase†	Dry wt (%)
Normal	7	327 (303-366)	20 (14-28)	48 (30-64)	16
Castrate 4 days	6	266 (168-318)	24 (18-33)	59 (53-64)	15
" 8 days	6	103 (75-140)	11 (7-13)	32 (25-48)	22
Testosterone 4 days	6	434 (376-487)	18 (17-21)	51 (38-76)	16
" 8 "	6	728 (608-881)	17 (12-23)	50 (42-62)	17

\* Pertains to enzyme values only. Gland weights and dry weights determined on 3 animals in each group.

† Expressed as Huggins-Talalay units per 100 mg dry tissue. Figures in parentheses indicate ranges.

the prostatic AIP-ase increases in concentration under the influence of androgen. The enzyme is localized in the epithelial elements of the gland, a positive phosphatase response being found at the basal and luminal borders of the epithelial cells. Since the androgen is trophic chiefly to the epithelium in this gland, causing an increased epithelial height, it is readily seen that androgenic stimulation should cause a relative increase in that amount of enzyme activity per unit weight of tissue.

*Discussion.* These data indicate that, contrary to previous belief,<sup>4</sup> the phosphatases in the prostate gland of the rat respond to androgen in a manner similar to that in the monkey and the dog. The quantitative data on the seminal vesicle presented here confirm the histochemical observations of Atkinson<sup>4</sup> and of Dempsey *et al.*<sup>5</sup> on the rat.

Davis, Meyer, and McShan<sup>10</sup> have shown that the succinic dehydrogenase (SDH-ase) and cytochrome oxidase in the prostate and seminal vesicle of the rat vary with the androgen supply in a manner somewhat similar to that shown here for the phosphatases. They found that in the castrate animal, the SDH-ase activity drops off as the weight of the glands decreases and returns when testosterone is administered. There is, however, one important difference, in the response of the two sets of enzymes studied. In both studies, dosage of testosterone used was sufficient to cause hypertrophy of the glands beyond the normal state. When this happened in the SDH-ase—cytochrome oxidase study, the en-

zyme activity rose beyond the level found in normal animals. In the case of the phosphatases, however, when the weight of the prostate was increased to more than twice that of the normal gland by 8 days of androgen treatment, the phosphatase activity did not rise beyond the normal level. The seminal vesicle under the same conditions hypertrophied to 70% above the normal weight, but the phosphatase activity did not rise above normal.

This phenomenon explains why Gutman and Gutman<sup>4</sup> were unable to show a response of the phosphatases to testosterone in their experiments; they were using adult, intact animals which presumably were at the maximal level of prostatic phosphatase activity before testosterone injections were begun.

The observations that the seminal vesicle is more sensitive to androgen deprivation than the prostate, morphologically<sup>11</sup> and with respect to oxidative enzyme activity<sup>10</sup> is borne out here again with respect to the phosphatases. While the seminal vesicle shows marked decrease in weight and phosphatase activity by the fourth day after castration, the prostate does not show a clear-cut response until sometime after this stage.

*Summary.* The acid and alkaline phosphatases of seminal vesicles and prostate glands of rats were estimated quantitatively in normal animals, castrates, and castrates receiving testosterone propionate.

In both glands the enzymes decrease in activity after castration, are restored to essen-

<sup>10</sup> Davis, J. S., Meyer, R. K., and McShan, W. H., *Endocrinology*, 1949, **44**, 1.

<sup>11</sup> Korenchevsky, B., Dennison, M., and Brossin, L., *Biochem. J.*, 1936, **30**, 558.

TABLE II.

Effect of Various Concentrations of Eserine and DFP on the Activities Displayed towards ECIA and Bch by Heated\* Human Plasma.

Inhibitor	Conc. of inhibitor (M)	Inhibition of Bch hydrolysis (%)	Inhibition of ECIA hydrolysis (%)
Eserine	$1 \times 10^{-6}$	97	99
	$1 \times 10^{-7}$	87	87
	$1 \times 10^{-8}$	51	52
	$1 \times 10^{-9}$	11	8
DFP	$8 \times 10^{-9}$	87	90
	$2.5 \times 10^{-9}$	43	43

\* Heated at 53°C for 90 minutes.

due to an enzyme or combination of enzymes distinct from the pseudo-ChE. That the eserine-sensitive residual ECIA hydrolysis by the heated plasma preparation was due to pseudo-ChE could be concluded from the following evidence: (a) various concentrations of eserine and DFP inhibited the activities towards ECIA and Bch to the same degree (Table II); (b) when hydrolysis was measured in the presence of a mixture of both substrates (ECIA and Bch) no addition of the separate activities was observed—rather, only an activity similar to that exhibited towards Bch alone was displayed; (c) exposure of the treated plasma to a temperature of 56–57°C for 30 minutes depressed the activities towards Bch and towards ECIA to the same degree (76% and 73% respectively).

(3) To demonstrate conclusively that the pseudo-ChE does not contribute to the eserine-insensitive portion of the hydrolysis of ECIA by crude tissue preparations, the activity of highly purified pseudo-ChE preparations towards ECIA was also investigated. Using a 9000-fold purified preparation of the pseudo-ChE obtained from horse serum,<sup>14</sup> the ratio activity towards ECIA (0.04M)

was found activity towards Bch (0.006M) to be about 1.5, as compared with a ratio of 130 with a sample of untreated horse serum. Moreover, although a low concentration ( $10^{-6}$ M) of eserine inhibits only a minute fraction of the activity of untreated horse serum towards ECIA, the activity towards ECIA of the pseudo-ChE purified from the same source is completely abolished by the same concentration of eserine. It is evident,

therefore, that the major portion of the hydrolysis of ECIA by horse serum is due to the presence of eserine-insensitive ali-esterases which are removed from the preparation upon extensive purification of the pseudo-ChE contained therein.

From the evidence obtained it can be concluded that the pseudo-ChE does hydrolyse ECIA but only at about  $1\frac{1}{2}$  times the rate at which it hydrolyses Bch and, further, its activity towards ECIA is inhibited just as effectively by eserine as is its activity towards Bch. While various crude enzyme preparations may exhibit extremely high activity towards ECIA—the ratio

activity towards ECIA activity towards Bch varying from 4 in some samples of human plasma to 130 in horse serum and over 1000 in rat plasma—only that small portion of the total hydrolysis which is inhibited by low concentrations of eserine can be attributed to a cholinesterase. The large eserine-insensitive portion of this activity would, on the other hand, seem to be due to a distinctly different enzyme, an ali-esterase. This ali-esterase, it might be noted, is probably not identical with the ali-esterases responsible for the eserine-insensitive hydrolysis of tributyrin and/or triacetin by tissue preparations, as is evidenced by differences in sensitivity to certain inhibitors (e.g., DFP) and by the fact that human plasma displays little or no eserine-insensitive activity towards these substrates although exhibiting a large eserine-insensitive activity towards ethyl chloroacetate<sup>15</sup> and certain substituted acetates of nitrophenol.<sup>15</sup>

<sup>14</sup> Strelitz, F., *Biochem. J.*, 1944, **38**, 86.

<sup>15</sup> Huggins, C., and Smith, D. R., *J. Biol. Chem.*, 1947, **170**, 391.



TABLE I.

Inhibition of the Activity of Human Plasma towards ECIA Brought about by Various Inhibitors.

Inhibitor	Conc. of inhibitor (M)	Inhibition of Bch hydrolysis (%)	Inhibition of ECIA hydrolysis (%)
Eserine	$2 \times 10^{-6}$	100	43*
	$2 \times 10^{-5}$	100	45
DFP	$4 \times 10^{-8}$	100	45
	$1 \times 10^{-6}$	100	44
	$1 \times 10^{-4}$	100	42

\* With 12 different samples of human plasma the eserine-sensitive portion of the ECIA hydrolysis varied from 7-45% (average ca 15%) of the total activity.

substrate is substituted for another, whether this substrate be a choline or a non-choline ester.

The observation of McNaughton and Zeller that ECIA is split with great rapidity in the presence of human serum or suspensions of guinea pig parotid gland has been confirmed and this rapid hydrolysis has similarly been found to be only slightly inhibited by  $10^{-5}$ M eserine. However, while these preparations have been shown<sup>10,11</sup> to contain relatively large amounts of pseudo-ChE, no evidence was presented by these authors<sup>8</sup> to substantiate their assumption that the high activity towards ECIA is not due, in part at least, to the presence of other esterases. To investigate the nature of the enzyme or enzymes responsible for the hydrolysis of ECIA by crude tissue preparations, the following experiments were carried out:

(1) To exclude the possibility that the low percentage inhibition of total ECIA hydrolysis by  $10^{-5}$ M eserine (a concentration which inhibits completely pseudo-ChE activity towards acetylcholine, benzoylcholine, tributyrin, etc.) might in some way be associated with the fact that the inhibition produced by this compound is 'competitive' and reversible<sup>12</sup> the crude preparation was incubated with diisopropyl fluorophosphonate (DFP), an irreversible inhibitor with very high affinity for pseudo-ChE.<sup>13</sup> In the presence of

$5 \times 10^{-8}$ M DFP, pseudo-ChE was completely inactivated, as indicated by the total absence of activity towards benzoylcholine (Bch), a substrate specific for the pseudo-ChE's of most mammalian tissues.<sup>10</sup> Despite the complete and irreversible inactivation of its pseudo-ChE, however, human plasma tested in the presence of this concentration of the inhibitor still displays a high activity towards ECIA. Moreover, the degree of inhibition of total ECIA hydrolysis produced by this concentration of DFP was almost identical with that observed with eserine in concentrations which also completely abolish the activity of human plasma towards benzoylcholine. Since evidently the portion of the hydrolysis of ECIA which is insensitive to eserine is also insensitive to DFP, the eserine-insensitive portion of the ECIA hydrolysis is most likely due to a type of esterase distinct from the enzyme responsible for the eserine-sensitive portion of the activity. This supposition is further confirmed by the fact that considerable increases in the concentration of either inhibitor fail to augment the inhibition appreciably (Table I), in contrast to the results which would be expected if only one enzyme, the pseudo-ChE, were responsible for the total activity displayed towards ECIA.

(2) To prevent any ali-esterases from participating in the activity displayed towards ECIA, preferential inactivation of these enzymes was effected by heating undiluted oxalated human plasma at 52-53°C for 60-90 minutes. Plasma treated in this manner still displayed 65-75% of the activity of the untreated plasma towards Bch and all of its residual activity towards ECIA was inhibited by eserine. These results demonstrate that the eserine-insensitive hydrolysis of ECIA is

<sup>10</sup> Mendel, B., Mundell, D. B., and Rudney, H., *Biochem. J.*, 1943, **37**, 473.

<sup>11</sup> Mendel, B., and Rudney, H., *Science*, 1943, **98**, 201.

<sup>12</sup> Straus, O. H., and Goldstein, A., *J. Gen. Physiol.*, 1943, **26**, 559.

<sup>13</sup> Hawkins, R. D., and Mendel, B., *Brit. J. Pharmacol.*, 1947, **2**, 173.

TABLE I.  
Effects of Imidazole Derivatives on the Growth of *K. pneumoniae*.

Substance tested	Concentration $\times 10^{-5}$ M	Turbidity readings, time in hr					
		14	16	18	20	22	24
Histamine $\cdot 2\text{HCl}$	50	9	34	96	130	130	130
	10	7	22	70	130	130	130
1 (+) Histidine $\cdot \text{HCl}$	50	50	104	130	130	130	130
	10	19	62	116	130	130	130
5-(4)-amino-4(5)-imidazolecarboxamide $\cdot \text{HCl}^*$	50	0	14	40	110	122	122
	10	0	4	15	60	116	126
Imidazole-4,5-dicarboxylic acid*	50	21	74	120	123	123	123
	10	6	38	100	130	130	130
Imidazole*	50	0	2	10	37	98	130
	10	0	1	6	25	86	128
p-Aminobenzoic acid 20 $\gamma$ /ml†		0	0	3	12	53	109
Control		0	0	4	19	66	112

\* 5(4)-Amino-4(5)-imidazolecarboxamide was obtained from Dr. John M. Buchanan. The other compounds were from Eastman Kodak Co.

† With 0.1 and 1.0  $\gamma$ /ml growth was of similar magnitude.

used in this study. The inocula, derived from 24-hour salt-glucose cultures, were washed once with and resuspended in phosphate buffer and diluted so that after inoculation each system contained approximately 50 viable cells per ml. All cultures were incubated at 37°C. The turbidity readings (filter No. 54, Klett-Summerson photoelectric colorimeter) represent averages of triplicate determinations.

**Micro-determination of Histamine and Histidine.** This method is an adaptation of the method of Macpherson<sup>1</sup> and was carried out in Klett tubes. The final volume of the reaction system was 6 ml and consisted of from 2 to 20  $\gamma$  of histamine or histidine per ml, 3 ml of water, 0.4 ml of sulfanilic acid in 10% hydrochloric acid solution, 0.4 ml of 5% sodium nitrite, and 1.2 ml of 30% sodium carbonate added after 30 minutes standing at room temperature. Colorimetric readings were made immediately with the use of the No. 54 filter. This permitted the determination of 2  $\gamma$  of either compound.

**5(4)-Amino-4(5)-imidazolecarboxamide.<sup>2</sup>** This substance was determined according to

the method of Bratton and Marshall.<sup>3</sup> This method carried out in Klett tubes permits the determination of 2  $\gamma$  of the compound.

**Results.** The results given in Table I clearly indicate the stimulatory effects of histidine, histamine, imidazole-4, 5-dicarboxylic acid, and 5(4)-amino-4(5)-imidazolecarboxamide. Histidine concentration of  $2 \times 10^{-5}$  M, and histamine,  $5 \times 10^{-5}$  M, also exercised definite stimulatory effects. Imidazole has but slight effect and p-aminobenzoic acid has no effect. Maximal growth and the logarithmic growth rates are approximately equal for all systems. At the times of maximal growths in the accelerated systems (18 to 20 hours), the turbidities were 5 to 10 times greater than that of the controls. The observed stimulatory effect is that of shortening the lag phase, and is not as apparent when large inocula are used.

During growth there was no measurable utilization of histamine or of 5(4)-amino-4(5)-imidazolecarboxamide. Histidine was utilized in proportion to growth. Histidine can serve as a sole source of carbon for growth in the absence of glucose while histamine cannot. For lack of a quantitative method, it was not determined whether or not imidazole-4,5-di-

<sup>1</sup> Macpherson, H. T., *Biochem. J.*, 1942, 36, 59.

<sup>2</sup> Shive, W., Ackermann, W. W., Bordon, M., Getzendaner, M. E., and Eakin, M. E., *J. Am. Chem. Soc.*, 1947, 69, 725.

<sup>3</sup> Bratton, A. C., and Marshall, E. K., *J. Biol. Chem.*, 1939, 128, 537.

Investigations with two 'irreversible' inhibitors—DFP as a preferential inhibitor of pseudo-ChE and tri-*o*-cresyl phosphate (TOCP) as a preferential inhibitor of ali-esterases<sup>16</sup>—showed that these compounds, unlike eserine, cannot be used as a general means of distinguishing pseudo-ChE and ali-esterase activities: certain ali-esterases, *e.g.*, the ali-esterase of rat plasma hydrolysing ECIA, exhibit abnormally high sensitivity to DFP, while others, *e.g.*, the ali-esterase of rat brain hydrolysing tributyrin, proved to be quite insensitive to TOCP. In other words, the portion of the total activity inhibited by low concentrations of DFP may not always be a reliable index of pseudo-ChE activity and similarly the portion of the total activity inhibited by TOCP may not always be a reliable criterion of ali-esterase activity. However, in all cases tested as yet the ali-esterases have proved to be much less sensitive to eserine than the cholinesterases of the same tissue. In fact, so far as is known, a concentration of  $2 \times 10^{-6}$ M eserine can be used to inhibit completely or almost completely the cholinesterase

activities in mammalian tissue preparations while leaving those of the ali-esterases unaffected.

*Summary.* Recent observations by McNaughton and Zeller on the high 'eserine-insensitive' activity displayed towards ethyl chloroacetate by various crude preparations of pseudo-ChE have been confirmed but the experimental results reported here fail to substantiate their conclusion that the activity of pseudo-ChE towards ECIA is insensitive to eserine. The evidence presented here has, in fact, indicated that the activity of any of the cholinesterases towards ethyl chloroacetate is inhibited by low concentrations of eserine, the remaining eserine-insensitive activity being due to ali-esterases. The value of eserine for distinguishing that portion of the hydrolysis of aliphatic esters which is due to the cholinesterases from that due to ali-esterases in crude tissue preparations has been re-affirmed. It would seem that of the three inhibitors tested (eserine, diisopropyl fluorophosphonate, tri-*o*-cresyl phosphate), only eserine can be relied upon to give a clear cut distinction between cholinesterase and ali-esterase activities in all cases.

<sup>16</sup> Mendel, B., and Mortimer, E. M., Report to National Research Council of Canada, October, 1944.

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## 17191. Histamine and Other Imidazole Compounds as Bacterial Growth Stimulators.\*

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In the course of a study it was observed that small amounts of histamine markedly stimulated the growth of certain bacteria. This report represents typical results of a comparative study of this effect with histamine and other derivatives of the imidazole nucleus.

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† Abbott Fellow in the Department of Bacteriology, 1948-1949.

*Materials and methods.* The culture medium was composed of NaCl (0.5%), MgSO<sub>4</sub> · 7H<sub>2</sub>O (0.02%), (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub> 0.1%, K<sub>2</sub>HPO<sub>4</sub> (2.4%), KH<sub>2</sub>PO<sub>4</sub> (0.6%), glucose (0.2%) and distilled water. The reaction of the medium was pH 7.0. It was passed through a sintered glass filter to sterilize. Solutions of the test substances in the salt-glucose medium were also sterilized by filtration. No significant drop in pH occurred during growth with this high phosphate buffer concentration. A stock strain of *Klebsiella pneumoniae* was

TABLE I.  
Effect of *Salmonella* Endotoxin on Intermediate Metabolites of the Rat Liver During Glycogen Synthesis. (Dose of endotoxin = 9 mg/100 g rat).

Metabolite	Normal starving	Normal fed glucose	Starving, inj. with endotoxin	Fed glucose and inj. with endotoxin
Glycogen	440	41,500	0	0
Glucose-1-PO <sub>4</sub>	86	68	15	10
Glucose-6-PO <sub>4</sub>	1,980	1,350	220	240
Fructose-6-PO <sub>4</sub>	200	80	10	10
Fructose-1-6-diphosphate	187	180	220	230
Pentose-PO <sub>4</sub>	130	128	74	30
Triose-PO <sub>4</sub> phosphorus*	17	31	30	75
Phosphopyruvic acid	68	15	0	0
Pyruvic acid	9.5	9.5	18	12
Lactic acid	37	148	310	390
Pyrophosphate phosphorus†	46	10	6.0	29
Phosphocreatine	90	1,470	500	800
Insoluble nucleic acid phosphorus‡	1,100	830	760	550

\* Values for triose phosphate phosphorus express the alkali labile phosphorus of the Ba soluble, alcohol-precipitable fraction.

† Values for pyrophosphate phosphorus represent the 7 minute phosphorus, obtained by hydrolysis of the Ba insoluble fraction in 1 N HCl.

‡ The nucleic acid phosphorus was determined by the trichloroacetic acid procedure of Schneider in the insoluble protein residue.<sup>4</sup>

this intermediate. On this basis the inhibition of glycogen synthesis could at least partially be explained by an inhibition of biological oxidation. This was already suggested by previous observations, which showed the decreased succinoxidase capacity of the tissues of rabbits injected with endotoxins.<sup>1</sup> The significance of the decrease of the insoluble nucleic acid phosphorus cannot be evaluated at present.

Since it is generally accepted that the main pathway of biological oxidation is by way of pyruvate oxidation,<sup>2</sup> it appeared to be of special interest to demonstrate pyruvic oxidase inhibition *in vitro*, which inhibition was suggested by the results obtained in animal tissues.

An alcohol precipitable fraction of meningococcal and *Salmonella* endotoxin proved to be a powerful inhibitor of pyruvate oxidation *in vitro*. The preparation of the inhibitory substance was carried out following a procedure used by Boivin and Mesrobian for extracting an antigenic carbohydrate-lipid complex from Gram negative bacteria (bibliography cited in <sup>6</sup>). The crude endotoxins were centrifuged at 4000 R.P.M. for 60 minutes at 0°. The supernatant mixture of soluble proteins was

precipitated by the addition of equal volumes of ice cold 10% trichloroacetic acid. The precipitate was sedimented by centrifugation and the supernatant neutralized to pH 7.0. This neutralized supernatant was dialyzed against running tap water for 18 hours. The final step was the addition of 8 to 10 volumes of cold 95% alcohol. A light flocculent precipitate settled down when the mixture was kept for 24 to 48 hours at 0°C. This alcohol precipitable fraction was centrifuged and after several washings with 95% alcohol was dried under a stream of warm air. The yield of this fraction was estimated to be 25 to 30 mg per liter of crude meningococcal endotoxin containing 1% solid material, and the yield from *Salmonella* endotoxin was 6 to 8 times greater. These materials could be resuspended in isotonic salt solution, yielding a slightly opalescent suspension.

Pyruvic oxidase was prepared by a method similar to that employed by Lehninger and Green.<sup>7,8</sup> Three grams of rat or rabbit liver were homogenized in 15 ml ice cold potassium

<sup>6</sup> Boor, A. K., and Miller, C. P., *J. Infect. Dis.*, 1944, 47, 75.

<sup>7</sup> Lehninger, A. L., *J. Biol. Chem.*, 1945, 437, 161.

<sup>8</sup> Green, D. E., Loomis, W. F., and Auerbach, V. H., *J. Biol. Chem.*, 1948, 389, 172.

<sup>5</sup> Vennesland, B., *Ann. Rev. Biochem.*, 1948, Vol. XVII, 227.

carboxylic acid and imidazole were utilized. In the case of histamine and 5(4)-amino-4(5)-imidazolecarboxamide, if utilized, the stimulatory effect must be due to amounts less than could be detected by the micromethods described above. Whether or not these effects have bearing on purine metabolism, or possess

a vitamine-like function are under investigation.

*Summary.* Histamine and certain derivatives of imidazole nucleus have been found in trace amounts to exercise stimulatory effects on the growth of *K. pneumoniae*.

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# 17192. Mechanism of Inhibition of Glycogen Synthesis by Endotoxins of *Salmonella aertrycke* and Type I Meningococcus.\*

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Endotoxins of *Salmonella aertrycke* and meningococcus were found to have a marked effect on carbohydrate metabolism as measured *in vivo*<sup>1,2</sup> and *in vitro*.<sup>2,3</sup> These observations indicated that the endotoxins act on both anaerobic and aerobic phases of carbohydrate metabolism. The present paper describes experiments, dealing with the effect of endotoxins *in vivo* and *in vitro* on the oxidation of pyruvate as related to glycogen synthesis.

*Experimental.* The inhibitory effect of crude *Salmonella* endotoxin on the synthesis of liver glycogen was studied in detail. The experimental conditions were similar to those previously described,<sup>2</sup> but the time of *in vivo* glycogen synthesis was extended to a period of 5 hours. Twelve rats were divided into 4 groups: the first group received no glucose and no toxin; the second group was given glucose by stomach tube but no toxin; while the third group received *Salmonella* endotoxin but no glucose; and the fourth received both glucose and endotoxin. The amount of *Salmonella* endotoxin was 9 mg per 100 g body weight,

a dose which was lethal for starving rats in 18 to 36 hours. Glucose was given 1 hour after the intraperitoneal injection of the endotoxin, and all animals were sacrificed 5 hours after glucose administration. The method of removal of liver samples and detailed analytical procedures for metabolic intermediates have been reported elsewhere.<sup>4</sup> Analytical results in each group of rats agreed from animal to animal within  $\pm 15\%$  for triose-phosphates, phosphopyruvate, hexose mono- and diphosphates and pyrophosphates, and within  $\pm 10\%$  for the rest of the intermediates. Results are summarized in Table I, where the analytical data are expressed in terms of micrograms of intermediates per gram of liver wet weight.

These analytical results confirmed previous findings<sup>2</sup> since even small doses of the endotoxin completely inhibited glycogen deposition. The lactic acid content of the livers of rats, injected with *Salmonella* endotoxin, was markedly increased, an effect which was similar to that observed in rabbits after the injection of endotoxins.<sup>1</sup> This finding itself indicated tissue anoxia, which, on the other hand, resulted in an inhibition of the function of the adenylic system. The inhibition of carbohydrate synthesis at the level of phosphopyruvate was suggested by the absence of

\* This investigation was supported by the U. S. Navy, Office of Naval Research, and the University of Chicago.

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<sup>1</sup> Kun, E., and Miller, C. P., *Proc. Soc. Exp. Biol. and Med.*, 1948, **67**, 221.

<sup>2</sup> Kun, E., *Proc. Soc. Exp. Biol. and Med.*, 1948, **68**, 496.

<sup>3</sup> Kun, E., *J. Biol. Chem.*, 1948, **761**, 174.

<sup>4</sup> Kun, E., and Abood, L. G., *Arch. Internat. Pharmacodyn. et Ther.*, (Gand, Belgium), 1949, **80**, 51.

TABLE I.  
Effect of Salmonella Endotoxin on Intermediate Metabolites of the Rat Liver During Glycogen Synthesis. (Dose of endotoxin = 9 mg/100 g rat).

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this intermediate. On this basis the inhibition of glycogen synthesis could at least partially be explained by an inhibition of biological oxidation. This was already suggested by previous observations, which showed the decreased succinoxidase capacity of the tissues of rabbits injected with endotoxins.<sup>1</sup> The significance of the decrease of the insoluble nucleic acid phosphorus cannot be evaluated at present.

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precipitated by the addition of equal volumes of ice cold 10% trichloroacetic acid. The precipitate was sedimented by centrifugation and the supernatant neutralized to pH 7.0. This neutralized supernatant was dialyzed against running tap water for 18 hours. The final step was the addition of 8 to 10 volumes of cold 95% alcohol. A light flocculent precipitate settled down when the mixture was kept for 24 to 48 hours at 0°C. This alcohol precipitable fraction was centrifuged and after several washings with 95% alcohol was dried under a stream of warm air. The yield of this fraction was estimated to be 25 to 30 mg per liter of crude meningococcal endotoxin containing 1% solid material, and the yield from Salmonella endotoxin was 6 to 8 times greater. These materials could be resuspended in isotonic salt solution, yielding a slightly opalescent suspension.

Pyruvic oxidase was prepared by a method similar to that employed by Lehninger and Green.<sup>7,8</sup> Three grams of rat or rabbit liver were homogenized in 15 ml ice cold potassium

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<sup>7</sup> Lehninger, A. L., *J. Biol. Chem.*, 1945, **437**, 161.

<sup>8</sup> Green, D. E., Loomis, W. F., and Auerbach, V. H., *J. Biol. Chem.*, 1948, **380**, 172.

<sup>5</sup> Vennesland, B., *Ann. Rev. Biochem.*, 1948, Vol. XVII, 227.

INHIBITION OF LIVER PYRUVIC OXIDASE BY  
AN ALCOHOL-PRECIPTABLE FRACTION OF THE  
ENDOTOXINS OF SALMONELLA AERTRYCKE AND  
TYPE 1 MENINGOCOCCUS

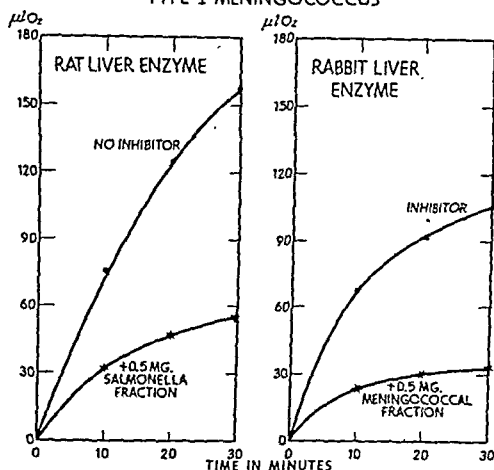


FIG. 1.

Each Warburg flask contained 300 mg enzyme, 0.5 ml 0.01 M adenosinetriphosphate, 10 mg sodium pyruvate as substrate, and 0.2 ml 20% sodium hydroxide in the center. The endotoxin fraction (0.5 mg) was suspended in 1 ml potassium medium. The final volume in each flask was made up to 3 ml. Oxygen consumption was measured at 37°C. The gas phase was air.

medium (containing 100 parts of 0.9% potassium chloride, + 21 parts 1.3% potassium bicarbonate, + 1 part 3.82% magnesium sulfate heptahydrate; to 120 ml of this mixture 1 ml of 2.1% potassium acid phosphate was added immediately before use). The homogenate was centrifuged and washed 3 times in the cold as described by Green<sup>8</sup> and finally resuspended to a volume, which contained 300 mg of original liver homogenate per ml. After the addition of adenosinetriphosphate and

sodium pyruvate, oxygen consumption was measured with and without the addition of 0.5 mg of the endotoxin alcohol-precipitable fraction. In the presence of this fraction the oxygen consumption, due to the addition of pyruvate, was invariably depressed.

It was of interest that the alcohol precipitable fraction of meningococcal endotoxin was very toxic to rabbits (0.5 to 1.0 mg injected intravenously produced death in 24 hours) while rats and mice were susceptible to the Salmonella fraction (70 to 150 μg injected intravenously were lethal to mice and rats in 16 to 24 hours).

A similar difference was observed *in vitro*: the pyruvic oxidase of the rabbit liver was inhibited by the meningococcal endotoxin fraction, while the rat liver enzyme was sensitive to the Salmonella endotoxin preparation.

**Summary.** *In vivo* and *in vitro* experiments indicated that the inhibition of pyruvate oxidation occurred in animals injected with bacterial endotoxins. The inhibitory factor was probably identical with the toxic "antigène complet" of Boivin and Mesrobianu. It is suggested that the inhibition of pyruvate oxidation plays an important role in the toxic effect of endotoxins. The exact mechanism of the inhibition of pyruvate oxidation and the identification of the inhibitor as well as the biochemical nature of other components of the endotoxins remains to be further investigated.

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# 17193. Effects of Certain Azo Dyes upon the Cockroach *Blattella germanica*.\*

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Very little is known about the effects of carcinogens upon insects. In the present study the cockroach *Blattella germanica* (L.) was fed crude or synthetic diets containing varying amounts of p-dimethylaminoazobenzene (DAB), m'-methyl-p-dimethylaminoazobenzene (m'DAB) or aminoazobenzene (AAB). The effects of the dyes upon growth rate, maturity, survival, ability to reproduce and the occurrence of internal lesions were determined. Newly-hatched nymphs from single egg sacs were divided into groups of 5-7 and kept in wire-screened test-tube cages<sup>1</sup> at a temperature of 27-32°C. Food and water were given *ad libitum* and were replaced weekly. The nymphs were weighed under CO<sub>2</sub> anaesthesia at 10, 20, and 30 days of age. The age at maturation, the ability to reproduce, and the fraction surviving were recorded. All surviving adults were eventually dissected and examined for abnormalities.

**Experimental.** In 3 preliminary experiments DAB was fed at 0.03, 0.06, 0.1 and 0.2%, and m'DAB was fed at 0.06 and 0.2% of a crude diet consisting of dog biscuits (Friskies) 94%, corn oil 4% and cholesterol 1 or 0.1%. Both the growth rate and reproductive performance of these insects remained essentially normal. The insects were dissected at ages varying from 54 to 151 days. At these times abnormalities of the digestive tract were seen in 37 of the 43 adults exposed to the

dyes, while none of 50 insects fed only the crude diet were affected similarly. A common condition was the occurrence of small black flecks in the walls of the esophagus, crop, mid-gut and hind-gut and in the tissue of the fat body. A characteristic finding in insects fed 0.1% DAB was a degeneration of the Malpighian tubules, which were sticky and tended to form clumps or nodules. In some cases small vesicles were present at the proximal ends of the tubules. In Experiment 1, 3 of 5 roaches fed 0.1% DAB were affected, in Experiment 2, 4 of 5 and in Experiment 3, 0 of 5. The entire digestive tracts of these affected insects were removed, imbedded by the dioxan-paraffin method, cut serially into sections 6-10 microns thick and stained with hematoxylin, with or without eosin counterstain. Photomicrographs were taken of representative lesions (Fig. 1-3).

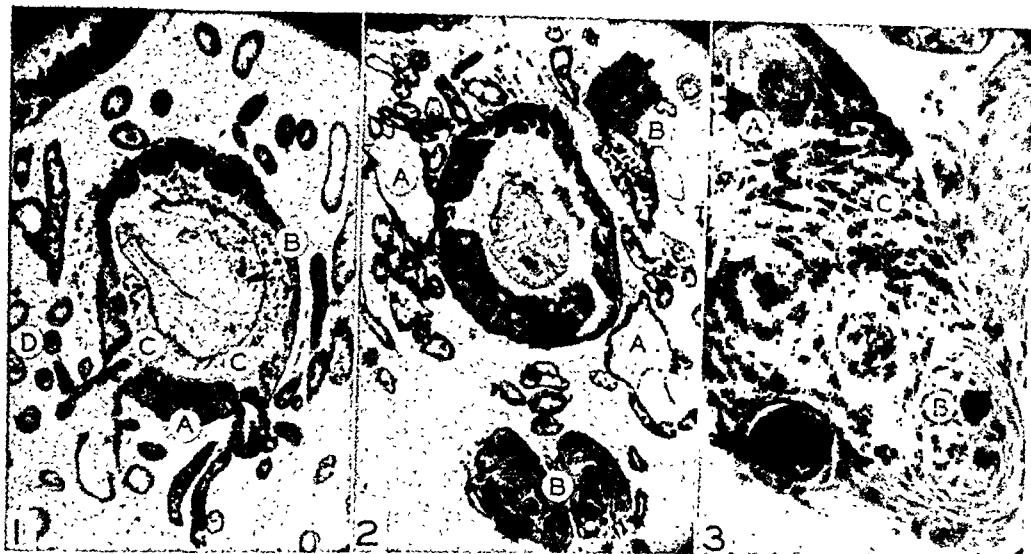
In a more detailed study, the potent carcinogens DAB and m'DAB were fed for long periods of time at a level of 0.2% in both crude and synthetic diets, and the non-carcinogen AAB was fed at 0.2% in a synthetic diet (Table II). One hundred and four newly-hatched nymphs from 4 egg sacs were distributed among 18 cages and fed the 6 diets shown in Table I. Growth rate, reproduction, and survival were measured, and the adults were dissected at ages varying from 112 to 173 days. Some of the F<sub>1</sub> nymphs from each group were kept on the same diets as their parents, and were killed for examination at 97-101 days of age. In addition to the experimental insects, a total of 127 adults previously fed a variety of crude and synthetic diets not containing azo dyes were dissected at ages varying from 53 to 143 days.

The growth rate, reproduction, and survival of the roaches were not greatly affected by the three azo dyes, although growth was retarded

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<sup>1</sup> Noland, J. L., Lilly, J. H., and Baumann, C. A., *Ann. Ent. Soc. Am.*, 1949, **42**, 63.





Cross-section of cockroach gut at the junction of mid-gut and hind-gut, showing lesions in the Malpighian tubules due to the feeding of azo dyes. A full description of this region as found in the normal cockroach is given by Miall and Denny.<sup>2</sup>

Fig. 1. Low Power. Cockroach previously fed 0.1% DAB for a short period of time. A—mid-gut. B—hind-gut. C—Origin of Malpighian tubules. D—Malpighian tubules in cross-section showing the beginning of degeneration.

Fig. 2. Low Power. Cockroach previously fed 0.1% DAB for a long time, showing the two characteristic types of lesions of the Malpighian tubules due to the azo dyes. A—Small vesicles formed by swelling of the tubules. B—Nodules formed by the fusion of adjacent tubules.

Fig. 3. High Power. Detail of a nodule shown in Fig. 2. A—Outlines of Malpighian tubules within nodule. B—Yellow granule of azo dye or its derivatives. C—Surrounding layers of hemocytes.

slightly when m'DAB was fed in the crude diet (age at maturity increased from 55 to 59 days) or DAB was fed in the synthetic diet (age at maturity increased from 49 to 64 days) (Table I). No consistent effect upon either reproduction or survival could be detected. The characteristic abnormality in insects fed the azo dyes in these experiments was the occurrence of large vesicles at one or more of the 6 points at which the Malpighian tubules arise from the intestine (see Fig. 1 and 2 for the location of these lesions). The vesicles ranged in size from one-fourth to 10 times the diameter of the mid-gut, and as many as 4 were observed in a single insect (Table II). The vesicles were usually thin-walled and filled with a light yellow fluid, but some were thick-walled and contained either a fluid or dark brown granules. In some individuals the proximal portions of adjacent Malpighian tubules were fused into nodules (Fig. 2 and 3). The extent of

vesicle formation did not appear to be affected by the type of basal diet fed. Of the 3 dyes, DAB appeared to cause the most lesions, followed by m'DAB which was fairly active, and AAB which was slightly active (Table II).

**Discussion.** It is evident from these experiments that the cockroach has a much higher tolerance for the carcinogenic azo dyes than has the rat or mouse. 0.06% DAB or m'DAB in the diet seriously retards growth and prevents reproduction in the rat<sup>5,6</sup> while more than three times this level (0.2%) of either

<sup>2</sup> Miall, L. C., and Denny, A., *The Cockroach*, London, 1886, 123.

<sup>3</sup> Noland, J. L., Lilly, J. H., and Baumann, C. A., *Ann. Ent. Soc. Am.*, in press, II.

<sup>4</sup> Noland, J. L., and Baumann, C. A., *Proc. Soc. Exp. Biol. and Med.*, 1949, **70**, 198.

<sup>5</sup> Miller, J. A., Miner, D. L., Rusch, H. P., and Baumann, C. A., *Cancer Res.*, 1941, **1**, 699.

<sup>6</sup> Giese, J. E., Miller, J. A., and Baumann, C. A., *Cancer Res.*, 1946, **6**, 337.

TABLE I.  
Growth Rate, Reproduction and Survival of Cockroaches Fed Various Azo Dyes.

Basal*	Diet supplement†	Wt at 30 days, mg	Age at maturity, days	Egg-sacs produced	Avg No. per egg-sac	Survival (fract.)
Crude	None	29	55	3	32	14/17
"	DAB	25	55	9	25	14/18
Synt.	"	31	64	1	29	11/18
Crude	m'DAB	20	59	5	28	9/12
Synt.	"	25	57	7	20	15/17
"	AAB	36	49	9	29	14/17

\* The crude basal diet consists of "Friskies" dog biscuits 95% and corn oil 5%. The synthetic basal diet is "synthetic diet V."<sup>3,4</sup>

† All supplements were fed at 0.2% of the diet. DAB = *p*-dimethylaminoazobenzene; m'DAB = *m*'-methyl-*p*-dimethylaminoazobenzene; AAB = aminobenzene.

TABLE II.  
Occurrence of Vesicles in the Malpighian Tubules of Cockroaches Fed Various Azo Dyes.

Diet*		Roaches dissected			Incidence of vesicles		
Basal	Supplement	Age, days	Sex M	F	% incidence	Avg No. per roach	Avg diam.†
First generation roaches.							
Crude	None	112-123	10	6	0	0	0
"	DAB	112-123	7	6	58	2.0	2.4
Synt.	"	112-123	6	3	89	2.1	2.5
Crude	m'DAB	112-123	6	4	30	1.3	1.2
Synt.	"	112-123	7	6	75	1.3	0.6
"	AAB	112-173	4	3	29	1.3	0.6
Second generation roaches.							
Crude	None	97	0	10	0	0	0
"	DAB	97	5	10	100	1.7	2.5
Synt.	"	97	4	11	87	1.7	1.2
Crude	m'DAB	97	7	8	53	1.8	0.7
Synt.	"	97	3	10	46	1.7	0.5
"	AAB	101	7	6	69	1.6	0.6
Unselected roaches.							
Crude	None	90	25	25	0	0	0
Fed 19 different synthetic diets		53-143	38	39	0	0	0

\* For composition of diets, see footnote to Table I.

† Expressed as the ratio of the diameter of the vesicle to that of the mid-intestine, which is normally 0.75 mm in diameter.

compound had little or no effect upon the growth rate of the roach. Another difference is the relative order of activity of the 3 compounds fed. For the production of liver tumors in rats, m'DAB is much more active than DAB, and AAB is quite inactive<sup>6,7</sup> while for the production of Malpighian tubule lesions in the roach, DAB appeared to be more active than m'DAB, and AAB was also somewhat active. It is of interest that in the rat,

tumors due to azo dyes are seldom found outside the liver<sup>8</sup> while in the roach, nodules or vesicles appeared only in the proximal region of the Malpighian tubules. Whether these latter lesions are tumors is doubtful; certainly no proof of autonomous growth is at hand.

An indication of the probable mechanism by which the lesions are formed was obtained by consecutive examination of serial sections

<sup>7</sup> Miller, J. A., and Baumann, C. A., *Cancer Res.*, 1945, 5, 227.

<sup>8</sup> Rusch, H. P., Baumann, C. A., Miller, J. A., and Kline, B. E., *A.A.A.S. Res. Conf. on Cancer*, 1945, 267.

of affected tissues. Apparently the azo dyes are absorbed from the gut into the hemolymph and are then taken up by the Malpighian tubules. If the tubules cannot excrete the dyes or their degradation products into the hind-gut, as is ordinarily done with other excretory substances,<sup>9</sup> the dyes or their derivatives will accumulate in the proximal portion of the tubules. Occlusion of the open ends of the tubules could lead to their swelling into vesicles (Fig. 2) as the absorption of fluid by the tubules continues. If the ends remain open, but the dyes stay within the tubules, local effects of the dye could cause the tubules to degenerate, become sticky and adhere to adjacent tubules. Hemocytes then accumulate around the affected areas to form nodules (Fig. 2 and 3). Wigglesworth<sup>10</sup> cites similar cases in which the hemocytes of insects en-

capsulated internal parasites, or walled off areas of infection or irritation. An alternative explanation of nodule formation would be that the nodules are tumors which were formed by the action of the azo dyes upon the Malpighian tubules. However, there appears to be no evidence at present which would lend support to this view.

**Summary.** The azo dyes *p*-dimethylaminoazobenzene (DAB), *m'*-methyl-*p*-dimethylaminoazobenzene (*m'*DAB) and aminoazobenzene (AAB) were fed to the roach *Blattella germanica* (L) at varying levels in both crude and synthetic diets. A dietary level of 0.2% of the dyes had little or no effect upon growth rate, survival or reproduction of the insects. However, characteristic lesions at the proximal ends of the Malpighian tubules occurred in roaches fed the dyes. DAB was the most active, *m'*DAB was next, and AAB was least active of the compounds fed.

<sup>9</sup> Wigglesworth, V. B., *Insect Physiology*, London, 1942, 310.

<sup>10</sup> Wigglesworth, V. B., *Insect Physiology*, London, 1942, 236.

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### 17194. Relation of Pantothenic Acid to White Blood Cell Response of Rats Following Stress.\*

MARY E. DUMM, PAUL OVANDO, PAUL ROTH, AND ELAINE P. RALLI.

From the Laboratories of Department of Medicine, New York University College of Medicine.

Recent work has emphasized the role of the adrenal cortex in controlling the number of lymphocytes in the circulating blood following various forms of stress. Corticosterone produced a lymphopenia when injected into either adrenalectomized or intact mice.<sup>1</sup> The injection of the adrenocorticotrophic hormone (ACTH) of the pituitary also produced a lymphopenia, provided the adrenals were present.<sup>1,2</sup> A deficiency of pantothenic acid causes lipid depletion and necrosis of the adrenal cortex.<sup>3-6</sup> Because of the importance

of pantothenic acid to the physiological integrity of the adrenal cortex and in view of the influence of the adrenal cortex on the number of white blood cells, experiments have been done to determine the effect of diets adequate and deficient in pantothenic acid on the response of the white cells of rats subjected to stress.

**Experimental.** Young male rats of the Long-Evans strain, bred in the laboratory,

\* This research was aided by a grant from the National Vitamin Foundation.

<sup>1</sup> Dougherty, T. F., and White, A., *Endocrinology*, 1944, **35**, 1.

<sup>2</sup> Reinhardt, W. O., Aron, H., and Li, C. H., *Proc. Soc. Exp. Biol. and Med.*, 1944, **57**, 19.

<sup>3</sup> Morgan, A. F., and Simms, H. D., *Science*, 1939, **89**, 565.

<sup>4</sup> Daft, F. S., and Sebrell, W. H., *U. S. Public Health Reports*, 1939, **54**, 2247.

<sup>5</sup> Ralli, E. P., and Graef, I., *Endocrinology*, 1943, **32**, 1.

<sup>6</sup> Deane, H. W., and McKibben, J. M., *Endocrinology*, 1946, **38**, 385.

TABLE I.

Supplements Added to 100 g Basal Diet (22% Vitamin-free Casein, 64% Sucrose, 9% Primex, and 5% NaCl-free Salt Mixture).

	Diet 100	Diet 1	Diet 14
Cod liver oil	1.9 ml	1.9 ml	1.9 ml
d, l- $\alpha$ -tocopherol acetate	145 mg	.0 "	.0 "
Thiamine chloride	1.0 "	.3 mg	.3 mg
Pyridoxine chloride	1.0 "	.3 "	.3 "
Nicotinic acid	10 "	.0 "	.0 "
p-Aminobenzoic acid	30 "	.0 "	.0 "
Riboflavin	2.0 "	.9 "	.9 "
Inositol	5.0 "	.0 "	.0 "
Choline chloride	100 "	.0 "	.0 "
Biotin	.05 "	.0 "	.0 "
Pteroylglutamic acid	.20 "	.0 "	.0 "
Calcium pantothenate	1.0 "	.0 "	43.6 "

were used. They were fed one of the 3 diets whose composition is shown in Table I. Diet 1, the pantothenate deficient diet, and Diet 14, the high pantothenate diet, have been used in previous experiments reported from this laboratory.<sup>5,7,8</sup> Diet 100 is a modification of a diet used by Emerson.<sup>9</sup> The rats consumed 10-15 g of Diets 14 and 100, and 6-10 g of Diet 1 daily.

**Experiment 1.** The response to stress of normal rats on a complete diet containing an adequate<sup>10</sup> but minimal quantity of calcium pantothenate was studied. Rats 80 days of age were placed on Diet 100 *ad lib.* and 1% NaCl as drinking water for 2 weeks. At the end of this time they were subjected to swimming in large stone crocks filled up to 4 inches of the top with tap water at 25°C. The rats were obliged to swim for 25 minutes. Just before swimming and at 2, 4, and 6 hours afterwards, tail blood was taken for total leucocyte counts and for blood smears. The total leucocytes were counted in the usual manner; estimation of the per cent lymphocytes was based on differential counts of at least 200 cells. The white blood cell response was studied in rats fasted for 17 hours before swimming and in rats allowed food up to the time of swimming. All animals were provided with food after swimming.

**Experiment 2.** Male rats 30 days of age were placed on the pantothenate deficient diet (Diet 1) and were given 1% NaCl as drinking water. After 25-30 days on the deficient diet, the animals were swum under the same conditions as the rats in Experiment 1 and the response of the white blood cells was followed.

**Experiment 3.** After 30 days on the deficient diet the rats were transferred to Diet 14, which provided them with about 4 mg of calcium pantothenate daily. After 4-12 days on this diet the stress experiments were repeated and the response of the white blood cells observed.

**Experiment 4.** Normal rats on Diet 100 and pantothenate deficient rats on Diet 1 were given 4 mg ACTH<sup>†</sup> per 100 g body weight intraperitoneally. Immediately before the injection of ACTH and at 1, 2, 3, 4, 6 and 8 hours afterward the total leucocytes and percentage leucocytes were determined as before. The animals were not fasted for this experiment.

**Results.** The response of the white blood cells following swimming or ACTH in the rats on the various diets is shown graphically in Fig. 1. The figures on the left summarize experiments in which food was allowed up to the time of stress, and on the right the response following a period of fasting is shown. Each point represents the average of counts on 12-21 rats.

<sup>7</sup> Ralli, E. P. *Endocrinology*, 1946, 39, 225.

<sup>8</sup> Damm, M. E., and Ralli, E. P., *Endocrinology*, 1948, 43, 283.

<sup>9</sup> Emerson, G., personal communication, 1948.

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*Experiment 2.* Male rats 30 days of age were placed on the pantothenate deficient diet (Diet 1) and were given 1% NaCl as drinking water. After 25-30 days on the deficient diet, the animals were swum under the same conditions as the rats in Experiment 1 and the response of the white blood cells was followed.

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## WHITE BLOOD CELL RESPONSE TO STRESS

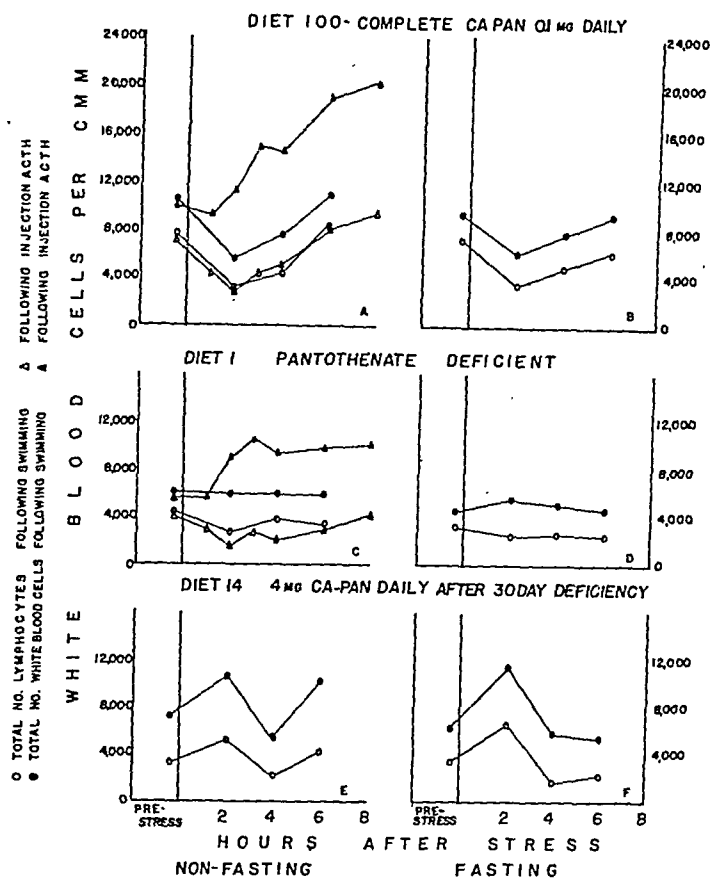


FIG. 1.

Effects of stress on response of white blood cells in rats on diets deficient in and supplemented with pantothenic acid.

The results of the experiments on normal rats maintained on Diet 100 are shown in Figure 1, A and B. This diet provided about 0.1 mg of calcium pantothenate daily. The experiments illustrate the marked lymphopenia which is known to occur when normal rats are subjected to stress.<sup>1</sup> The lymphocyte response was the same after swimming as after the injection of ACTH. Two hours after swimming or ACTH the lymphocyte counts had decreased to about half the initial values. After 6 hours the number of cells had returned to their initial values. The total leucocytes also decreased following swimming, but increased to about twice their initial value following ACTH. The results of the swimming experiment with fasted normal rats (Fig. 1, B) were similar to those in

fed rats, although the initial counts were slightly lower.

The results in the pantothenic deficient rats are shown in Fig. 1, C and D. The total number of white blood cells and lymphocytes was lower in these rats than in the rats on the adequate diet. Both swimming and the injection of ACTH were followed in these animals by a small, but probably significant ( $p < .05$ ), decrease in lymphocytes after 2 hours. The lymphocyte response was more pronounced after ACTH than after swimming. After ACTH the total number of white blood cells increased to about twice their original level but there was no significant change in the total number of white blood cells after swimming in the fed animals and only a small increase in the fasted rats.

*Discussion.* The data presented show that pantothenic acid can modify the response of the white blood cells in rats subjected either to a natural form of stress or to ACTH. In rats on normal diets both swimming and ACTH were followed by a significant decrease in the number of lymphocytes, occurring within 2 hours. In rats that had been fed a diet deficient in pantothenic acid for 3-4 weeks the lymphopenia following stress was largely abolished. Lipid depletion of the fascicular and reticular zones of the adrenal cortex has been reported after a period of pantothenic acid deficiency<sup>5,6</sup> and these are the zones probably responsible for the secretion of the corticosterone-like substances.<sup>11</sup> Dougherty and White<sup>4</sup> have shown that the injection of corticosterone in mice will produce lymphopenia. It may well be that the lipid depletion of the adrenal cortex produced by pantothenic acid deficiency was responsible for the decreased lymphocyte response to stress in the rats.

The addition of very large doses of calcium pantothenate to the diet of deficient rats restored the white blood cell response to stress. The nature of the response under these conditions was not that of the normal rat. The lymphopenia was delayed until the 4th hour after stress and was preceded by an increase in the lymphocytes at the 2nd hour. This occurred in both the fasted and non-fasted rats.

While general malnutrition is associated with adrenal cortical changes, it seems un-

likely that this was an important factor in the results presented here. Deane *et al.*<sup>6,12</sup> have reported that neither pyridoxine nor riboflavin deficiency produced lipid depletion in the adrenal cortex of the rat and that inanition alone did not exhaust the adrenal as rapidly as a deficiency in either pantothenic acid or thiamine.

The decrease in the response of the white blood cells to stress in the animals on diets deficient in pantothenic acid is consistent with the changes in the adrenal cortex associated with the deficiency and is further evidence that pantothenic acid plays an important role in maintaining adrenal cortical function.

*Summary.* The responses of white blood cells and lymphocytes following swimming and following ACTH were compared in rats on a complete diet, in rats on a pantothenic acid deficient diet, and following swimming only in rats recovering from a period of pantothenic acid deficiency. A typical lymphopenia occurred 2 hours after either swimming or ACTH in rats on the complete diet. This response was partially abolished following either swimming or ACTH in the rats on the pantothenate deficient diet. In rats recovering from pantothenate deficiency, there was an increase in lymphocytes 2 hours after swimming followed by a decrease at 4 hours. These results are interpreted as a reflection of the changes in the adrenal cortex induced by pantothenic acid deficiency.

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<sup>12</sup> Deane, H. W., and Shaw, J. H., *J. Nutrition*, 1947, **34**, 1.

<sup>11</sup> Deane, H. W., and Greep, R. O., *Am. J. Anat.*, 1946, **79**, 117.

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# 17195. An Improved Method for the Microbiological Assay of Growth Factors on Paper Chromatograms.\*

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The use of paper partition chromatography in the separation of microbial growth factors has recently been reported by Winsten and Eigen<sup>1,2</sup> and Cuthbertson and Smith.<sup>3</sup> These workers placed the developed chromatogram strips on nutrient agar seeded with the test organism. After incubation, zones of growth of the organism were noted along the site of each strip, indicating the presence of various growth factors. The relative concentration of these factors was estimated by the size of the zones.

The method of Winsten and Eigen<sup>1</sup> has been modified so that the chromatogram may be assayed both qualitatively and quantitatively for microbial growth factors using an improved technic. The improved method involves a direct test-tube microbiological assay of the developed chromatogram rather than the more tedious agar plate procedure. A description of the improved method, as used

in the microbiological assay of paper chromatograms on which crystalline vitamin B<sub>12</sub>,<sup>4</sup> thymidine,<sup>†</sup> and injectable liver extract<sup>†</sup> were placed, is given in this report. *Lactobacillus leichmannii* (ATCC 4797) was used as the assay organism.

According to Shive, Ravel and Harding,<sup>4</sup> vitamin B<sub>12</sub> and thymidine are interchangeable growth factors for *L. leichmannii* (ATCC 4797). Shive, Eakin, Harding, Ravel and Sutherland<sup>5</sup> have reported that thymidine may be present in amounts as high as 1% in some experimental injectable liver extracts. The presence of thymidine, therefore, may produce erroneous results in assaying liver preparations and other materials for vitamin B<sub>12</sub>. By applying the procedure described in this paper, thymidine interference may be avoided.

**Method.** Whatman No. 1 filter paper (46 cm × 57 cm) was used in a chromatogram chamber similar to that described by Consden, Gordon and Martin.<sup>6</sup> A line was drawn across the paper 10 cm from the top edge. Along this line were placed small dots 3 cm apart. A known volume of the solution to be tested was placed on each dot with a microliter pipette using graded levels of from 1 to 10 microliters. In general each level was placed on from 2 to 4 dots. Evidence that the results could be duplicated was obtained by this means.

In no case was the spot of liquid allowed to spread more than 1 cm in diameter. Spreading was prevented by applying the liquid to the paper in portions of 3 microliters or less and allowing the liquid to evaporate between

\* This work was undertaken in cooperation with the Office of Naval Research, Navy Department, Washington, D.C., and was aided by grants from the Nutrition Foundation, New York City, the Cerophyl Laboratories, Kansas City, Mo.; Commercial Solvents Corporation, New York City; the Cooperative G.L.P. Exchange, Ithaca, N. Y., and the Western Condensing Co., San Francisco. The technical assistance of Betty F. Brown and Diana M. Cameron is acknowledged.

<sup>1</sup> Winsten, W. A., and Eigen, E., *Proc. Soc. Exp. Biol. and Med.*, 1948, **67**, 513.

<sup>2</sup> Winsten, W. A., and Eigen, E., *J. Biol. Chem.*, 1949, **177**, 989.

<sup>3</sup> Cuthbertson, W. F. J., and Smith, E. Lester, *Biochem. J.*, 1949, **44**, No. 2 (*Proc. Biochem. Soc.*, v).

<sup>†</sup> The crystalline vitamin B<sub>12</sub> used in this work was kindly supplied by Dr. E. Lester Smith of Glaxo Laboratories, England, and the thymidine by Dr. William Shive of the University of Texas. The injectable liver extract was Lilly's extract containing 15 U.S.P. units per ml.

<sup>4</sup> Shive, W., Ravel, J. M., and Harding, W. M., *J. Biol. Chem.*, 1948, **176**, 991.

<sup>5</sup> Shive, W., Eakin, R. E., Harding, W. M., Ravel, J. M., and Sutherland, J. E., *J. Amer. Chem. Soc.*, 1948, **70**, 2299.

<sup>6</sup> Consden, R., Gordon, A. H., and Martin, A. J. P., *Biochem. J.*, 1944, **38**, 224.

TABLE I.

Results of Assay of Paper Chromatograms Containing Injectable Liver Extract, Vitamin B<sub>12</sub> and Thymidine.

Substance assayed	Amt. placed on paper	Location of growth by number of 2 cm section	Distance to solvent front in cm	Recovery in growth zone in %
Crystalline vitamin B <sub>12</sub>	0.1 mγ	1	44.5	98.3
	0.2 "	1	37.0	104.4
	0.3 "	1	42.5	98.8
Liver extract, 15 units/ml	0.00015 unit	1	44.2	106.7
	0.0003 "	1	43.5	100.9
	0.0015 "	1	37.0	100.0
	0.0030 "	1	37.0	100.0
Thymidine	0.5 γ	4-7	35.4	—*
	1.0 γ	4-8	35.4	—
	1.4 γ	4-8	35.4	—
	2.1 γ	4-8	34.8	—
Vitamin B <sub>12</sub> + thymidine	0.3 mγ	1	43.4	100.0
	0.2 γ	5-7	—	—
Liver extract + thymidine	0.00015 unit	1	35.4	103.3
	0.5 γ	4-7	—	—
Liver extract + thymidine	0.0045 unit	1	35.4	96.0
	1.4 γ	3-8	—	—

\* Determination was omitted because sufficient thymidine was not available to prepare standard curves.

applications. An electric fan facilitated rapid evaporation. The spots were air dried and the paper was then placed in the chromatogram chamber with the upper edge immersed in the solvent trough.

The solvent used was n-butyl alcohol saturated with water. This was placed in the trough and also in each of two beakers at the bottom of the chamber to insure complete saturation of the chamber with the solvent vapors. The chromatographic procedure was carried out at room temperature. After 24 hours, or when the solvent had advanced approximately 40 cm, the paper was removed and suspended in a hood in front of a fan until all traces of solvent had disappeared.

The paper was then cut lengthwise in strips 3 cm wide so that each strip contained the material from one spot. These strips were then cut crosswise into sections 2 cm wide. The small sections of filter paper were each placed in test tubes, 10 ml of a newly developed medium<sup>7</sup> added and the tubes steamed

15 minutes. This was followed by inoculation with *L. leichmannii* (ATCC 4797) using a drop inoculator. Drop inoculation was found necessary since loop inoculation resulted in fraying of the filter paper which caused increased turbidity. After 16 hours incubation, growth was measured turbidimetrically in a Coleman Spectrophotometer set at 650 millimicrons.

When carrying out quantitative assays, the values for the standard curves were obtained by placing graded amounts of the standard solution of crystalline vitamin B<sub>12</sub> on 2 x 3 cm sections of dried, butanol-treated filter paper using a microliter pipette and assaying these along with the developed chromatogram.

The presence of filter paper had no significant effect upon the growth of the organism. Pieces of filter paper ranging in size from 1 x 3 cm to 6 x 3 cm were tested and no appreciable differences in growth noted.

*Results and Discussion.* In Table I are given some typical results using the improved procedure in the assay of chromatograms for vitamin B<sub>12</sub> and thymidine. The method was

<sup>7</sup> Peeler, H. T., Yacowitz, Harold, and Norris, L. C., unpublished results.

found to be sensitive to 0.1 millimicrograms of vitamin B<sub>12</sub> present in the developed chromatogram. No measureable destruction of vitamin B<sub>12</sub> was noted in the chromatographic procedure.

In the assay of the liver extract and crystalline vitamin B<sub>12</sub> all the growth was confined to the first 2 cm section of the chromatograms. The remaining sections of the chromatogram failed to promote any growth response, indicating that the liver extract used in our experiments contained little, or no, thymidine or other growth factors. Interference from thymidine, therefore, did not occur when assaying the liver extract at a level of 0.0045 unit, or less, per tube.

In preliminary work it is necessary to assay each cross sectional piece of filter paper in the entire length of the chromatogram. Once the range of  $R_f$  values of any substance has been accurately determined, it is only necessary to test the section known to contain that particular substance. For example, after separating thymidine from vitamin B<sub>12</sub>, only the first 2 cm section of the chromatogram needs to be tested to obtain the vitamin B<sub>12</sub> content of the substance being assayed.

The  $R_f$  value of vitamin B<sub>12</sub> was found to

$$\frac{\uparrow R_f \text{ (rate of flow)}}{\text{Distance from starting line to center of growth zone}}$$

Distance from starting line to solvent front

Distance from starting line to solvent front

be less than 0.04 while the large thymidine zone had an average  $R_f$  value of 0.33 and varied between 0.31 and 0.37. This thymidine  $R_f$  value differs from that reported by Hotchkiss<sup>8</sup> who obtained an  $R_f$  value of 0.51 using the same solvent but in the presence of gaseous ammonia. Winsten and Eigen<sup>9</sup> reported a double zone of growth for thymidine with the principal zone having an  $R_f$  value of 0.54 and a minor zone with an  $R_f$  value of 0.41. These latter workers were not able to state unequivocally which of these zones was due to thymidine.

The improved procedure has also been used successfully with the ascending solvent technic described by Williams and Kirby.<sup>9</sup> It appears applicable to the study of unidentified growth factors required by other microorganisms as well as *L. leichmannii* (ATCC 4797) and in the study of growth inhibitors and antibiotics.

**Summary.** A simple, quantitative method for the microbiological assay of filter paper chromatograms has been described. Some possible applications have also been pointed out.

<sup>8</sup> Hotchkiss, R. D., *J. Biol. Chem.*, 1948, **175**, 315.

<sup>9</sup> Williams, R. J., and Kirby, H., *Science*, 1948, **107**, 481.

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## 17196. Duodenal Ulcers Produced on a Diet Deficient in Pantothenic Acid.\*

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It is well known that various nutritional essentials are concerned with the maintenance of normal epithelial tissue. As part of a program concerning nutritional deficiencies and gastrointestinal epithelium, we have reported the finding of marked antral gastritis on

calcium deficient diets.<sup>1</sup> Similar, less marked and less constant lesions were also reported on thiamine deficiency. Under various conditions of inanition the fundic mucosa will show rather typical areas of hemorrhage.<sup>2</sup> Previously, many papers have appeared on the

\* This investigation was supported by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service.

<sup>1</sup> Zucker, T. F., Berg, B. N., and Zucker, L. M., *J. Nutrition*, 1945, **30**, 301.

<sup>2</sup> Zucker, T. F., Berg, B. N., and Zucker, L. M., *J. Nutrition*, 1945, **30**, 319.

involvement of nutritional deficiencies in the production of hyperplasia of the forestomach epithelium of rats.<sup>3</sup> In none of these cases, even when modifications were introduced to accentuate the process, have penetrating lesions been observed which could in the narrower sense of the word be designated as ulcers or which resembled typical ulcers as they occur spontaneously in man.

Further observations have shown that on a diet deficient in pantothenic acid penetrating ulcers are produced in rats. The diet contained all the known nutritional factors necessary for growth except pantothenic acid, and had the following composition per 100 g of diet: vitamin "free" casein (Labco) 18.0; cerelose 71.9; salt mixture 6.1 (Ca 1.0, P 0.55); cottonseed oil 2.0; celluloflour 2.0. Incorporated in the oil were 10 mg alpha tocopherol and 1500 international units of vitamin A as carotene. The celluloflour carried: thiamine HCl and pyridoxine HCl 1 mg each; riboflavin 2 mg; niacin 4 mg and 2-methylnaphthoquinone 0.5 mg.

Out of a larger group covering various age ranges, 45 animals have been autopsied: 23 older rats with an initial age averaging 394 (271-620) days and 22 younger rats starting at 42 to 105 days. The average experimental time for the older animals was 100 (67-140) days and for the younger ones 125 (87-185) days. As a rule the animals were autopsied when they showed severe weakness or an abrupt loss in weight.

Typical signs of pantothenic acid deficiency consisting of porphyrin deposit on the hair, degeneration, necrosis and hemorrhage in the adrenal cortex and fatty infiltration of the liver appeared in varying degree in all of the rats. Characteristic new findings were atrophy of the duodenal mucosa which was present in every animal, and duodenal ulcers which were encountered in 60% of the rats. The rest of the gastrointestinal tract was unaffected. Several hundred normal control rats and animals kept on diets deficient in other B factors failed to show similar changes in the duodenum.

Areas of atrophy appeared as smooth de-

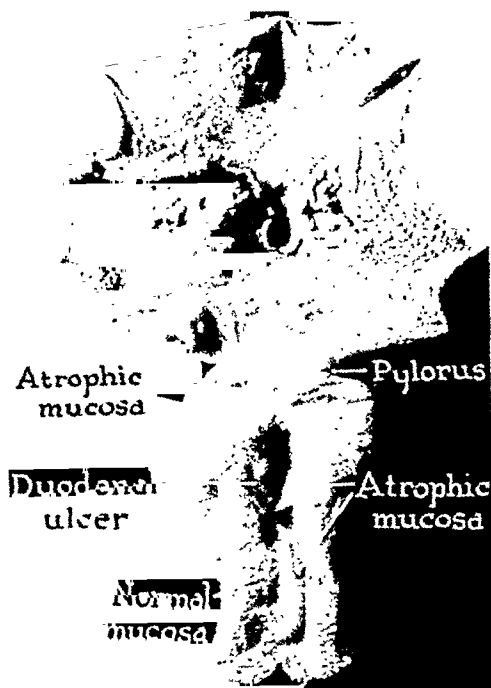


Fig. 1.  
Large perforated duodenal ulcer. Base formed by connective tissue and adherent pancreas. Also shown, are areas of mucosal atrophy.

pressions of various shapes and sizes. Sometimes the greater part of the surface was involved except for a few remaining islands of intact mucosa. Microscopically, villi were absent and were replaced by a flat layer of low cuboidal or squamous-like epithelium. Lesser degrees of atrophy were also noted. Here the villi were shortened or resembled simple tubular glands.

Ulcers have thus far been seen chiefly in atrophic mucosa. They occurred in 17 older rats and in 10 younger ones; in 17 instances multiple ulcers were present. Grossly and microscopically the lesions corresponded to the so-called acute and chronic ulcers of humans. Acute lesions extending for a variable distance through the different layers of the duodenal wall were found in all of the 27 animals. In 6 rats (5 belonging to the older group) co-existing large perforated defects sealed by dense connective tissue and adherent pancreas were also observed. (Fig. 1). A fibrinopurulent peritonitis due to perforation was en-

<sup>3</sup> Berg, B. N., *Am. J. Path.*, 1942, 18, 49.

countered twice. Hemorrhage from eroded vessels in the fibrous base of ulcers was the cause of death in 2 animals.

In swine, Wintrobe and associates have described atrophy and ulcers in the colon.<sup>4</sup> They also reviewed signs of pantothenic acid deficiency in other species. No duodenal lesions occurred among their reported findings.

Whether the experimentally produced ulcers will acquire significance for the study of human ulcers remains to be seen. The possibility that the latter condition is caused directly by dietary pantothenic acid deficiency is remote considering the exceedingly wide distribution of this factor. To base a working hypothesis on these observations it would be

necessary to adduce evidence for abnormal paths of pantothenic acid metabolism. In rats, for instance, with adequate pantothenic acid in the diet but with prevention of intestinal synthesis of folic acid and biotin, a pantothenic acid deficiency in the tissues develops.<sup>5</sup> It is relieved by feeding folic acid and biotin. Orienting studies are under way to test out possibilities of pantothenic acid being involved in the human duodenal ulcer. In the meantime, however, the findings herein reported should be of value in elucidating the various steps of the pathological process which go into the formation of a duodenal ulcer, particularly the early stages which are seldom seen in human material.

<sup>4</sup> Wintrobe, M. M., Follis, R. H., Jr., Alcayaga, R., Paulson, M., and Humphreys, S., *Bull. Johns Hopkins Hosp.*, 1943, **73**, 313.

<sup>5</sup> Wright, L. D., and Weleh, A. D., *Science*, 1943, **97**, 426.

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## 17197. Neutralizing Antibody Against Viruses of the Encephalomyocarditis Group in the Sera of Wild Rats.

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The encephalomyocarditis (EMC),<sup>1</sup> MM,<sup>2</sup> Columbia-SK<sup>3</sup> and Mengo encephalomyelitis<sup>4</sup> viruses have been shown recently to be immunologically indistinguishable,<sup>5,6</sup> hence, they are apparently strains of the same agent. The viruses of the EMC family are now known to have a world-wide distribution, occurring in North America,<sup>1-3</sup> the Philippines,<sup>7</sup> and

Africa,<sup>4</sup> but the natural history of the disease and sources of human infection are still obscure.

On at least two occasions viruses of the EMC group have been demonstrated to be pathogenic for man. Neutralizing antibodies against EMC virus have been shown to appear in the convalescent sera of a group of American soldiers who contracted a mild febrile illness colloquially designated as "Three-Day Fever"<sup>7</sup> in Manila in 1945-46. In addition, acute encephalomyelitis caused by Mengo virus occurred in a laboratory worker at Entebbe, Africa.<sup>4</sup>

We were led to consider the possibility that the wild rat might serve as a reservoir for viruses of the EMC type for a number of reasons:

Mice, hamsters and cotton rats are highly susceptible and succumb to even small doses

<sup>1</sup> Helwig, F. C., and Schmidt, E. C. H., *Science*, 1945, **102**, 31.

<sup>2</sup> Jungeblut, C. W., and Dalldorf, G., *Am. J. Pub. Health*, 1943, **33**, 169.

<sup>3</sup> Jungeblut, C. W., and Sanders, M., *J. Exp. Med.*, 1940, **72**, 407.

<sup>4</sup> Dick, G. W. A., Best, A. M., Haddow, A. J., and Smithburn, K. C., *Lancet*, 1948, **2**, 286.

<sup>5</sup> Warren, J., Smadel, J. E., and Russ, S. B., *J. Immunol.*, 1949, in press.

<sup>6</sup> Dick, G. W. A., *J. Immunol.*, 1949, in press.

<sup>7</sup> Smadel, J. E., and Warren, J., *J. Clin. Invest.*, 1947, **26**, 1197.

TABLE I.  
Neutralizing Antibody Against EMC Virus in Sera of Wild Rats.\*

Locale	No. tested	Result†		
		Negative	Positive	% positive
British Columbia	22	22	0	0
California	100	87	13	13.0
Florida	93	84	9	9.7
Georgia	57	51	6	10.5
Louisiana	9	9	0	0
Maryland	43	38	5	11.6
Mich. (Detroit)	11	11	0	0
Mississippi	49	6	43	87.0
New Mexico	6	6	0	0
North Carolina	2	2	0	0
South Carolina	21	21	0	0
Texas	23	16	7	30.5
Wash. (Seattle)	6	6	0	0

\* Practically all the wild rats were *Rattus norvegicus*; some from California were *R. alexandrinus*, and all from South Carolina were cotton rats (*Sigmodon hispidus*).

† Sera containing sufficient antibody to protect against at least 100 minimal lethal doses of virus were considered positive, while those containing less than this amount were regarded as negative.

of virus injected peripherally.<sup>2-5</sup>

In contrast, the albino rat undergoes only an inapparent infection following a massive inoculum although considerable virus persists in the central nervous system<sup>3,5,8</sup> and the circulation.<sup>9</sup> The susceptibility of wild rats has not been determined but it was thought that it might be similar to the albino species.

One of the main foci of cases of Manila "Three-Day Fever" occurred in a camp located in a park which previously had been used by Japanese troops as a garbage dump. Although not mentioned in the original report of the outbreak, one patient whom we were able to interview recalled that rats were very numerous and cases of rat bite were occurring in this area.

**Methods.** The EMC, MM and Mengo viruses were maintained by intracerebral transfers in mice. The histories of the strains used in the present work were published in detail recently.<sup>6</sup> Rodent sera were collected in various regions of the country and shipped to this laboratory;\* approximately half were refrigerated during transit. Neutralization tests were performed in 12-14 g albino Swiss

mice using equal quantities of fresh infected mouse brain (appropriately diluted in distilled water) and undiluted serum. The virus-serum mixtures were incubated for 1 hour at 37.5°C after which 0.1 ml amounts of each mixture were inoculated intraperitoneally into each of 5 mice which were observed for 14 days. At the end of this time the neutralization index was calculated as the antilog of the difference of the LD 50% of the virus suspension in the control mixed with distilled water and in the test proper mixed with rat serum.

**Experimental.** Neutralization tests have been performed with sera from 442 wild rats (mainly *Rattus norvegicus* and *R. alexandrinus*) collected in various regions of the United States and Canada. Eighty-three of these had demonstrable antibody against EMC virus, i.e., neutralized at least 100 M.L.D.'s of virus, and the majority of the positive sera possessed sufficient antibody to protect against 10,000 or more M.L.D.'s of virus.

The findings summarized in Table I show

<sup>8</sup> Powell, H. M., Jamieson, W. A., and Culbertson, C. G., Proc. Soc. Exp. Biol. and Med., 1948, 68, 80.

<sup>9</sup> Warren, J., and Russ, S. B., unpublished experiments.

\* This study was made possible through the generous cooperation of the following persons, who supplied us with rodent sera: Doctors G. C. Brown, D. Coburn, D. Davis, M. Frobisher, Jr., F. A. Humphreys, J. V. Irons, A. S. Lazarus, E. H. Lennette, L. Llewellyn, A. Morris, J. G. Rempel, V. R. Saurino, and M. Shaffer.

that the preponderance of sera examined came from rats caught in California, Florida, Georgia, Maryland and Mississippi, and that the proportion of positive sera among the rats from different regions varied considerably. For example, 43 of 49 specimens obtained from rural areas of Mississippi had antibody, whereas, only 6 of 57 from Georgia gave positive results. Certain counties within a state provided a greater proportion of positive sera than others. Thus, 11 of 44 samples from Orange County in southern California neutralized the virus, whereas, only 2 of 48 from San Diego County did so. Nine of the 93 sera examined from Florida gave positive results and 5 of these were among the five sera obtained from the town of Dania. It may be noted that it was in this area that the virus of encephalomyocarditis was originally recovered<sup>1</sup> from a chimpanzee held captive on an animal farm. Georgia supplied 6 positive sera and 5 of these were from rodents trapped in Wayne County. It would appear from the present data that the incidence of antibody against EMC virus is highest in rats caught in certain southern regions of the United States.

Twenty-two of the rat sera which were capable of neutralizing the EMC strain were also tested for their protective effect against the MM and Mengo strains. As was anticipated from earlier work the sera neutralized all 3 viruses to about the same extent.

EMC neutralization tests were performed on 69 sera obtained from various wild animals<sup>†</sup> which were caught in the same areas of California and Maryland that had yielded positive rat sera. (Table II). These included specimens from 4 gophers, 15 opossums, 9 rabbits, 18 squirrels, 2 chipmunks, 8 marmots, 2 meadow mice, 2 raccoons, 3 skunks and 6 domestic cats. None of these sera were capable of neutralizing even small amounts of

TABLE II.  
Absence of Neutralizing Antibody Against EMC Virus in Sera of Rodents Other than the Rat.

Location	Species	No. tested	No. positive
Calif.	Gopher	4	0
	Opossum	13	0
	Rabbit	5	0
	Squirrel	18	0
	( <i>C. beecheyi</i> )		
Md. (Patuxent)	Chipmunk	2	0
	Marmot	8	0
	Meadow mouse	2	0
	Opossum	2	0
	Rabbit	4	0
	Raccoon	2	0
	Skunk	3	0
	Domestic cat	6	0

virus, *i.e.*, 50 M.L.D.'s.

**Discussion.** The EMC group of viruses appear to be ubiquitous, being widely distributed geographically and occurring in a number of host species. This should make one cautious in interpreting studies in which these viruses are apparently recovered from clinical material. Critical analysis of all the data is now required before one can assume that the agent found in passage animals bears an etiological relation to the disease in a given patient. Similarly, in view of present knowledge, "variant" strains encountered during serial transfer in animals such as the "high Lansing" mutant of poliomyelitis virus<sup>8</sup> should be thoroughly investigated for possible relation to the EMC group.

**Summary.** Sera from an appreciable number of 442 wild rats trapped in several widely separated areas of the United States contained neutralizing antibodies against the EMC group of viruses. The incidence of positive sera varied considerably in different geographic regions with the highest rate (87%) occurring in rats from the state of Mississippi.

Similar serological studies on other species of small wild animals failed to provide evidence of the occurrence among them of natural infection with the EMC viruses.

<sup>†</sup> We are indebted to Drs. E. H. Lennette, State Department of Public Health, Berkeley, Calif., and J. A. Morris, Patuxent Research Refuge, Laurel, Md., for these sera.

## 17198. Relief from Pruritus Following upon Administration of Adenylic Acid.

ANTONIO ROTTINO. (Introduced by H. G. Albaum.)

*From the Hodgkin's Disease Research Laboratory,\* Department of Medicine, St. Vincent's Hospital, New York City.*

In December, 1948, muscle adenylic acid was administered to a group of patients suffering from Hodgkin's disease in the hope that it might possibly have a beneficial influence upon their physical energy,<sup>†</sup> depletion of which is one of the disabling effects of this disease. Though the experiment proved a failure in this respect, a totally unexpected and gratifying result ensued: the only two patients of the group who had pruritus reported that this had completely disappeared. In an effort to establish whether or not this was mere coincidence the co-operation of colleagues<sup>‡</sup> was enlisted and medication extended to include persons afflicted with pruritus of diverse etiology.

In all, 36 patients were treated with the sodium salt of adenosine-5-monophosphate,<sup>§</sup> injected intramuscularly in a solution of 20 mg in 1 cc of water. The optimum dosage had to be determined by trial and error. Massive doses—100 mg hourly for 6 consecutive hours for a period of 11 days—was given to 4 patients (Nos. 1, 12, 28 and 35 in the table) and produced negative results on pruritis in all except case No. 28. Dosage of 20 mg hourly for 5 consecutive hours over a period of 3 days yielded fairly uniform results, including response by Case No. 1, who had not responded to the massive doses; Cases 12 and 35 were not retreated. Approximately half of

the patients responded to the therapy within 24 hours, the others in from 2 to 7 days.

In 5 instances the pruritus was associated with diabetes (4 cases under insulin control and one as yet untreated), 9 with Hodgkin's disease, one with carcinoma of the ovary, one with dermatitis due to hair-dye sensitivity, 18 were idiopathic, and 2 occurred post partum. In almost every instance the pruritus was severe (inducing in some of the sufferers talk of suicide) and had previously been subjected to numerous therapies: salves, injections, x-ray, ultra violet rays, etc. Distribution was general in 20 instances, limited to anus or genitalia in 12 instances, to the legs, feet and hands in 4 instances. Skin of many of the patients was excoriated, in some instances severely so.

*Results.* Results were negative for 6 patients and positive for 30. There were 9 cases of complete subsidence, 14 of marked improvement, 5 of moderate improvement, and 2 of mild improvement. Not enough time has yet elapsed to make possible the assembling of statistics as to how enduring these results may be. Five patients (2, 28, 29, 30, 36) have remained free of recurrence or maintained the state of improvement from discontinuance of therapy to date, this period varying from one to four months; others suffered recurrence within 24 hours to one month. Three (16, 28, 29) who suffered recurrence and were retreated reacted as favorably to the remedication as to the initial medication.

The only toxic symptom—a sensation of inability to breathe freely—was short-lived and experienced by those receiving massive dosage (1, 12, 28, 35).

*Discussion.* Considering the fundamental importance of adenylic acid to biologic mechanisms such as glycolysis, the Krebs cycle, muscle contraction, etc., it is surprising that so little is known concerning the

\* Supported in part by grants from The National Cancer Institute of The U. S. Public Health Service, The American Cancer Society, and The Damon Runyon Memorial Fund.

† This was done at the suggestion of Dr. Kurt Stern, Brooklyn Polytechnic Institute.

‡ Drs. Lloyd Craver, A. Susinno, Richard Kennedy, O. Canizares, A. Shapiro, J. Corr, W. Starkard, and F. Jost, to whom we express our sincere appreciation.

§ The preparation used was "My-B-Den," made and supplied by Ernst K. Biscoff Co., Inc.



TABLE I.

Summary of Clinical Data on 36 Patients with Pruritus Who Were Treated with Adenylic Acid.

Pruritus						Results produced by Administration of My-B-Den		
Pt. No. and age	Sex	Etiology	Distribution	Severity	Duration	Relief	Appeared, day	Duration
1-41	F	Idiopathic	Vulva	4+	7 mo.	4+	2nd	1 wk*
2-35	F	"	"	4+	4 wk	4+	3rd	4 mo.*
3-30	F	"	"	4+	11 yr	0	—	—
4-56	F	"	"	4+	1 yr	4+	3rd	2 wk
5-60	F	"	"	4+	15 yr	3+	1st	2 wk*
6-74	M	"	Anus	4+	4 mo.	3+	1st	2 wk*
7-56	F	"	"	3+	3 wk	3+	1st	3 days
8-43	M	"	"	4+	8 yr	4+	2nd	1 wk*
9-42	M	"	"	4+	3 yr	3+	1st	1 wk*
10-39	M	"	"	4+	12 yr	3+	1st	1 wk*
11-56	M	"	Generalized	4+	6 mo.	4+	1st	3 wk*
12-35	F	"	"	4+	30 yr	0	—	—
13-87	F	"	"	4+	ys	0	—	—
14-45	F	"	"	4+	6 mo.	2+	3rd	1 wk
15-43	F	"	"	4+	1 yr	2+	1st	No follow-up
16-22	M	"	"	3+	12 yr	3+	2nd	2 wk
17-67	M	"	"	4+	5 mo.	2+	3rd	2 wk
18-69	F	"	"	4+	5 yr	2+	4th	3 wk
19-30	F	Postpartum	"	4+	2 mo.	3+	1st	3 wk
20-32	F	"	"	4+	4 days	3+	7th	1 wk
21-62	F	Cancer	"	4+	6 mo.	3+	1st	3 wk*
22-64	F	Hair dye sens.	"	4+	10 days	0	—	—
23-65	M	Diabetes	Feet	4+	6 mo.	3+	1st	3 wk
24-86	F	"	Generalized	2+	ys	1+	3rd	2 wk
25-60	F	"	Vulva	4+	4 mo.	2+	2nd	†
26-55	F	"	"	4+	9 mo.	3+	1st	2 wk*
27-67	F	"	Legs	2+	2 mo.	4+	3rd	3 mo.
28-39	M	Hodgkins	Generalized	4+	1 yr	4+	1st	5 wk
29-39	M	"	Hands	3+	1 yr	4+	6th	1 mo.*
30-49	M	"	Generalized	4+	1 wk	4+	3rd	1 mo.*
31-48	F	"	"	4+	6 yr	0	—	—
32-43	M	"	"	3+	1 mo.	3+	3rd	2 wk
33-52	F	"	"	4+	1½ yr	1+	1st	1 wk*
34-39	M	"	Legs	3+	7 mo.	3+	1st	1 wk*
35-40	F	"	Generalized	4+	3 yr	0	—	—
36-42	F	"	"	3+	1 mo.	3+	2nd	4 wk*

\* Indicates patient was still free of pruritus at time this table was made.

† Only with medication. 20 mg hourly for five consecutive hours per day since May 7, '49.

4+ indicates complete relief from pruritus.

clinical disorders resulting from pathologic-physiologic disturbance involving metabolism of adenylic acid and related substances.

We feel that the results which were obtained with adenylic acid are too consistent to be attributable to chance and that these results may be interpreted to mean that pruritus is one indication of adenylic acid deficiency. Adenylic acid has previously been used therapeutically<sup>1-6</sup> but so far as we know no one has pointed out the relationship between its

administration and subsidence of pruritus. Beneficial effects on skin disorders have been

3 Carlström, B., and Olle Lövgren, *Acta Medica Scandinavica*, 1942, 110, 230.

4 Dietrich, S., and Schwiegl, H., *Dtsch. med. Wchnschr.*, 1934, 60, 967.

5 Herbrand, W., *Dtsch. med. Wchnschr.*, 1937, 63, 1841.

6 Holyroyd, F. J., *West Virginia Med. J.*, 1940, 36, 261.

7 Rothmann, H., *Rev. Gastroenterol.*, 1942, 9, 117.

8 Spies, T. D., Bean, W. B., and Vilter, R. W., *Ann. Int. Med.*, 1940, 13, 1616.

9 Vilter, R. W., Bean, W. B., and Spies, T. D., *J. Lab. and Clin. Med.*, 1942, 27, 527.

1 Bean, W. B., *Med. Clin. N. A.*, March 1943, 27, 483.

2 Carlström, B., and Olle Lövgren, *Acta Medica Scandinavica*, 1943, 115, 568.

alluded to but not expanded upon.<sup>3</sup> We have not hitherto concerned ourselves particularly with this specialized field and can only state that several of our patients made voluntary mention of the fact that their skin had become softer and less dry and that excoriations due to scratching had healed upon subsidence of the pruritus some whose pruritus had not entirely disappeared found that scratching failed to produce the excoriation which had been the result previous to treatment.

Though there is much yet to be done clinically and in the laboratory before the full implications of adenylic acid administration to human beings can be completely evaluated,

we feel that the results to date are sufficiently good to make broader experimentation desirable, and hence to warrant passing on our experiences to other workers.

*Summary.* Thirty-six patients suffering from pruritus of diverse etiology were treated with adenylic acid. In thirty instances there was a subsidence of the pruritus ranging from complete to mild. So far we have been able to find in the rather extensive literature no reference to the beneficial effect of adenylic acid upon pruritus.

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### 17199. Chemotherapy of Leukemia. IV. Effect of Folic Acid Derivatives on Transplanted Mouse Leukemia.\*

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Since the first report of promising results in the clinical treatment of acute leukemia with 4-amino-pteroylglutamic acid,<sup>1</sup> this drug<sup>2,3</sup> and two other related compounds<sup>3,4</sup> have been reported to be active against certain strains of transplanted mouse leukemia.

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<sup>†</sup> Fellow of The American Cancer Society, recommended by the Committee on Growth of The National Research Council.

<sup>1</sup> Farber, S., Diamond, L. K., Mercer, R. D., Sylvester, R. F., Jr., and Wolff, J. A., *New England J. Med.*, 1948, **238**, 787.

<sup>2</sup> Law, L., Abstract, Cancer Research, 1949, in press.

<sup>3</sup> Burchenal, J. H., Burchenal, J. R., Kushida, M. N., Johnston, S. F., and Williams, B. S., *Cancer*, 1949, **2**, 113.

<sup>4</sup> Burchenal, J. H., Bendich, A., Brown, G. B., Elion, G. B., Hitchings, G. H., Rhoads, C. P., and Stock, C. C., *Cancer*, 1949, **2**, 119.

These clinical and experimental findings suggested the advisability of screening a large number of compounds related to pteroylglutamic acid (PGA) against transplanted mouse leukemia. This series included not only compounds closely related in structure to folic acid, but also pyrimidines, pteridines and purines. The results of the preliminary testing of 90 such compounds are herewith reported.

*Method.* The technic for evaluation of the chemotherapeutic activity of a given drug by means of its ability to prolong the survival time of mice with transmitted leukemia has been described previously.<sup>3</sup>

In a typical experiment, 240 mice of the inbred Akm stock were injected intraperitoneally with 0.1 cc of a saline suspension of leukemic spleen so diluted that 0.1 cc contained 1,000,000 cells. Leukemia Ak 4,<sup>5</sup> a relatively acute strain, was used in these par-

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<sup>5</sup> Burchenal, J. H., Biedler, J. L., Nutting, J., Stobbe, G. D., to be published.

TABLE I.  
Compounds Showing Definite Chemotherapeutic Activity Against Leukemia Ak 4.

Survival time (days)														
Compound	Dose mg/kg	Wt change (gr.)				Untreated				Treated				% increase treated
		Untreated†		Treated*	No. Mice	Range	Mean	S.D.	No. Mice	Range	Mean	S.D.		
4-Amino-2,10-methyl- pteroylglutamic acid	3	+1.5	+0.5		19	11-19	13.4	±1.78		10	28-39	30.1	±3.08	125
	3	+2.3	-0.7		20	10-16	12.4	±1.5		7	31-37	34.6	±1.84	179
	3	+1.9	+2.9		19	11-16	13.2	±1.57		8	26-45	31.9	±5.65	142
	3	+1.9	-1.2		20	11-18	13.5	±1.66		9	21-32	24.9	±3.57	84
	3	+3.7	+3.1		20	11-19	13.3	±1.78		8	30-43	34.2	±5.44	157
	3	+4.9	+2.4		20	10-15	12.7	±1.76		10	20-40	29.3	±3.94	131
	3	+2.3	+1.1		20	10-14	12.1	±1.26		17	20-36	27.0		123
4-Amino-9-methyl pteroylglutamic acid	3	+1.7	+3.5		20	10-14	11.7	±1.12		6	19-29	24.5	±3.74	109
	4	+1.9	+1.8		20	11-18	13.5	±1.66		9	22-28	24.2	±2.57	79
	4	+2.9	+2.6		19	11-17	12.6	±1.70		7	19-43	29.3	±6.95	133
	4	+2.5	+5.5		20	10-18	11.7	±1.85		7	21-32	26.0	±4.29	122
	4	+3.3	+1.1		19	11-16	12.9	±1.47		6	33-41	37.1	±3.18	188
4-Amino-9,10-dimethyl pteroylglutamic acid	3	+1.7	+2.7		20	9-14	11.5	±1.24		10	22-34	29.5	±3.91	156
	5	+1.9	+1.0		20	11-18	13.5	±1.66		5	28-35	31.0	±2.68	129
	5	+2.5	+2.4		20	10-18	11.7	±1.85		5	32-38	34.2	±2.04	192
	3	+3.3	+0.9		19	11-16	12.9	±1.47		6	32-43	36.1	±3.58	180
	3	+1.7	-1.7		20	10-14	11.7	±1.12		6	28-42	34.2	±3.73	192
	3	+0.3	+0.5		16	10-14	12.2	±1.19		9	26-32	27.8	±1.81	128
2,6-Diaminopurine	90	+1.7	+0.9		20	10-16	12.0	±1.32		8	17-31	23.7	±4.44	97
	90	+0.8	+1.0		20	10-15	11.7	±1.74		9	19-26	22.5	±2.50	92
	90 × 2 75 × 8	-0.5	+1.4		20	10-14	11.3	±1.28		8	21-31	26.1	±3.85	131
	100	+2.3	-1.4		19	11-16	13.3	±1.87		10	20-40	28.6	±6.22	115
	90	+2.1	-0.5		19	9-16	10.7	±1.83		18	12-24	18.4	±3.94	72
	100	+1.0	-0.6		20	10-33	14.5	±4.50		9	20-31	24.4	±3.38	68

\* Weight change calculated as the difference between the

\* Weight change calculated as the difference between the initial weight and that two weeks later.  
† Weight change calculated as the difference between the initial weight and that one week later.  
S.D. = Standard deviation.

ticular experiments. Forty-eight hours later, these mice were divided into comparable groups of 10 mice each (2 sets of untreated controls, one set of controls treated with a standard compound of known activity, 4-amino- $N^{10}$ -methyl-pteroylglutamic acid, and 21 sets of mice treated with unknown compounds). Compounds were given intraperitoneally in maximum tolerated doses 3 times weekly for 10 doses. Water soluble compounds were dissolved in saline. Substances insoluble in water were usually suspended in 5% gum arabic in saline. The results of treatment with an unknown substance were compared with those obtained with the standard compound, 4-amino- $N^{10}$ -methyl-PGA which has previously been shown to possess a high degree of chemotherapeutic activity<sup>3</sup> against Ak 4 leukemia. Maximum tolerated dosage was used throughout in an attempt to procure the maximum effect. The mice were observed for the development of leukemia and autopsied at death. If gross evidence of leukemia was not conclusive, microscopic sections were taken. The rationale behind the various steps of this technic has been outlined in previous reports.<sup>6</sup>

**Results.** The derivatives of pteroylglutamic acid which show a definite chemotherapeutic effect against Ak 4 leukemia are listed in Table I. The compounds included here are only those which show approximately a doubling of the survival time of the treated animals as compared with the untreated controls. Experiments in which these compounds have been evaluated are listed in detail. Further data on two compounds previously reported<sup>3,4</sup> (4-amino- $N^{10}$ -methyl-PGA and 2,6-diaminopurine) are shown in Fig. 1. Table II includes those compounds which have shown a suggestive effect by increasing the average survival time approximately 50%. Table III lists the compounds which have shown no evidence of chemotherapeutic activity after at least one satisfactory test.

**Discussion.** In man, some leukemias do and some do not respond to antifolic therapy and, similarly, in the mouse not all strains of

TABLE II. Compounds Showing Slight to Moderate Chemotherapeutic Activity Against Leukemia Ak 4.

Compound	Dose mg/kg	Survival time (days)												
		Wt change (gr.)				Untreated				Treated				% increase treated
		Untreated†		Treated		No. Mice	Range	Mean	S.D.	No. Mice	Range	Mean	S.D.	
4-Amino-pteroyl- aspartic acid	30	+3.7	+4.6†	20	11-19	13.3	±1.78	10	15-25	18.1	±3.18	36		
	30	+2.4	+2.7*	18	8-15	12.2	±1.61	8	17-28	21.5	±3.74	76		
	30	+1.9	+2.4†	20	11-18	13.5	±1.66	10	14-32	18.4	±5.07	36		
	30	+2.3	-0.9*	20	10-16	12.4	±1.50	9	15-26	20.2	±4.05	63		
	30	+3.3	+0.6*	19	11-16	12.9	±1.47	9	15-53	25.5	±11.23	98		
4-Amino-3',5'-dibromo- pteroylglutamic acid†	60	+1.5	+1.2*	20	9-16	12.5	±1.87	9	14-33	19.3	±5.4	54		
	70	-0.9	+1.9†	19	10-22	13.5	±2.76	7	14-22	18.1	±2.54	34		
4-Amino-pteroyl threonine	200	+0.3	+1.3†	15	10-14	12.2	±1.19	10	14-27	19.5	±4.46	60		
	200	+3.5	+4.4*	19	10-13	11.0	±1.24	9	15-22	19.0	±1.88	73		
4-Amino-pteroyl- glutamic acid	0.3	+3.7	+2.8*	20	11-19	13.3	±1.78	8	15-25	20.0	±3.24	50		
	0.3	+3.3	+2.5*	19	11-16	12.9	±1.47	9	22-34	29.1	±3.24	126		

\* Burchenal, J. H., Lester, R. A., Riley, J. B., and Rhoads, C. P., *Cancer*, 1948, 1, 399.

TABLE I.  
Compounds Showing Definite Chemotherapeutic Activity Against Leukemia Ak 4.

Compound	Dose mg/kg	Survival time (days)											
		Wt change (gr.)						Treated					
		Untreated†		Treated*	No. Mice	Range	Mean	S.D.	No. Mice	Range	Mean	S.D.	% increase treated
		Untreated	Treated										
4-Amino-N,10-methyl- pteroylglutamic acid	3	+1.5	+0.5	19	11-19	13.4	±1.78	10	28-39	30.1	±3.08	125	
	3	+2.3	-0.7	20	10-16	12.4	±1.5	7	31-37	34.6	±1.84	179	
	3	+1.9	+2.9	19	11-16	13.2	±1.57	8	26-45	31.9	±5.65	142	
	3	+1.9	-1.2	20	11-18	13.5	±1.66	9	21-32	24.9	±3.57	84	
	3	+3.7	+3.1	20	11-19	13.3	±1.78	8	30-43	34.2	±4.18	157	
	3	+4.9	+2.4	20	10-15	12.7	±1.76	10	20-40	29.3	±5.44	131	
4-Amino-9-methyl pteroylglutamic acid	3	+2.3	+1.1	20	10-14	12.1	±1.26	17	20-36	27.0	±3.94	123	
	3	+1.7	+3.5	20	10-14	11.7	±1.12	6	19-29	24.5	±3.74	109	
	4	+1.9	+1.8	20	11-18	13.5	±1.66	9	22-28	24.2	±2.57	79	
	4	+2.9	+2.6	19	11-17	12.6	±1.70	7	19-43	29.3	±6.95	133	
	4	+2.5	+5.5	20	10-18	11.7	±1.85	7	21-32	26.0	±4.29	122	
	4	+3.3	+1.1	19	11-16	12.9	±1.47	6	33-41	37.1	±3.18	188	
4-Amino-9,10-dimethyl pteroylglutamic acid	3	+1.7	+2.7	20	9-14	11.5	±1.24	10	22-34	29.5	±3.91	156	
	5	+1.9	+1.0	20	11-18	13.5	±1.66	5	28-35	31.0	±2.68	129	
	5	+2.5	+2.4	20	10-18	11.7	±1.85	5	32-38	34.2	±2.04	192	
	5	+3.3	+0.9	19	11-16	12.9	±1.47	6	32-43	36.1	±3.58	180	
	3	+1.7	-1.7	20	10-14	11.7	±1.12	6	28-42	34.2	±3.73	192	
	3	+0.3	+0.5	16	10-14	12.2	±1.19	9	26-32	27.8	±1.81	128	
2,6-Diaminopurine	90	+1.7	+0.9	20	10-16	12.0	±1.32	8	17-31	23.7	±4.44	97	
	90	+0.8	+1.0	20	10-15	11.7	±1.74	9	19-26	22.5	±2.50	92	
	90 × 2	-0.5	+1.4	20	10-14	11.3	±1.28	8	21-31	26.1	±3.85	131	
	75 × 8	+2.3	-1.4	19	11-16	13.3	±1.87	10	20-40	28.6	±6.22	115	
	90	+2.1	-0.5	19	9-16	10.7	±1.83	18	12-24	18.4	±3.04	72	
	100	+1.0	-0.6	20	10-33	14.5	±4.50	9	20-31	24.4	±3.38	68	

\* Weight change calculated as the difference between 4

\* Weight change calculated as the difference between the initial weight and that two weeks later.  
† Weight change calculated as the difference between the initial weight and that one week later.  
S.D. = Standard deviation.



TABLE III.  
Compounds Showing No Evidence of Chemotherapeutic Activity Against Leukemia Ak 4.

Compound	Dose, mg/kg
<i>Pyrimidines</i>	
2-Amino-4-methylpyrimidine	750
2-Amino-4-(4'-arsonophenylamino)pyrimidine	15
2-Amino-4-methyl-5-acetylpyrimidine	64
2-Amino-4,5-dimethylpyrimidine	250
2-Amino-4,6-diacetylaminopyrimidine	175
2-Amino-4-hydroxy-5-(2',4'-dichlorophenoxy)pyrimidine	1000
2-Amino-4-hydroxy-5,6-dimethylpyrimidine	250
2-Amino-4-hydroxy-5-p-chlorophenoxy-6-methylpyrimidine	1000
2-Amino-5-bromo-6-hydroxypyrimidine	250
2,4-Diamino-5-methylpyrimidine	75
2,4-Diamino-5-(2', 4'-dichlorophenoxy)pyrimidine	250
2,4-Diamino-5,6-dimethylpyrimidine	35
2,4-Diamino-6-methylpyrimidine	125
2,4-Diamino-6-hydroxypyrimidine	450
2,5-Diamino-4,6-dihydroxypyrimidine	150
2,4-Dihydroxy-5-chloroacetamidopyrimidine	50
2,4-Dihydroxy-5,6-diaminopyrimidine	100
2,6-Dihydroxy-5-nitropyrimidine	150
2,6-Dihydroxy-5-bromopyrimidine	300
2,6-Dihydroxy-5-aminopyrimidine	250
2,6-Dihydroxy-4,5-diaminopyrimidine	15
2,4,6-Trihydroxypyrimidine	35
2,4,5,6-Tetrahydroxypyrimidine	250 × 2 125 × 7
2-Mercapto-4-hydroxypyrimidine	100
2-Mercapto-4-hydroxy-5-methylpyrimidine	1000
2-Mercapto-3-o-tolyl-4,6,6-trimethylpyrimidine	350
2,4,6-Trichloropyrimidine	15
2-Chloro-4-dimethylamino-6-methylpyrimidine	0.75
2-Methyl-4-hydroxy-5-ethoxymethylpyrimidine	750
1-Butyl-2-hendeeyl-1,4,5,6-tetrahydroxypyrimidine	4
<i>Hexahydropyrimidines</i>	
1,3-Bis(1,3-dimethylbutyl)-5-nitro-5-methylhexahydropyrimidine	750
1,3-Bis(1,3-dimethylbutyl)-5-nitro-5-ethylhexahydropyrimidine	300
1,3-Bis(2-ethylhexyl)-5-amino-5-methylhexahydropyrimidine	35
1,3-Diisopropyl-5-amino-5-methylhexahydropyrimidine	125
1,3-Dibenzyl-5-nitro-5-methylhexahydropyrimidine	750
1,3-Dibenzyl-5-amino-5-methylhexahydropyrimidine	32
1,3-Di-p-tolyl-5-amino-5-methylhexahydropyrimidine	75
<i>Pteridines</i>	
2,4-Diaminopteridine	100
2,4-Diamino-6-methylpteridine	200 × 6 100 × 1
2,4-Diamino-7-methylpteridine	60
2,4-Diamino-6-p-carboxyanilinomethylpteridine*	125
2,4-Diamino-6-N-methyl-p-carboxyanilinomethylpteridine	125
2,4-Diamino-6,7-dimethylpteridine	50
2,4-Diamino-6,7-dihydroxypteridine	500
2,4-Diamino-6,7-diphenylpteridine	100
2,4-Diamino-6,7-dicarboxypteridine	250
2,4-Diamino-6,7-bis(4-aminophenyl)pteridine	500
2,4-Diamino-6,7-bis(p-sulfonomethylaminophenyl)pteridine	400
2,4-Diamino-5,7-dihydroxypyrimido(4,5-e)pteridine	300
2-Amino-4-hydroxypteridine	1000 × 1 667 × 3
2-Amino-4-hydroxy-6-methylpteridine	15
2-Amino-4,5,7-trihydroxypyrimido(4,5-e)pteridine	300
2,4,6,7-Tetrahydroxypteridine	10
2,4-Dihydroxy-6,7-dimethylpteridine	250
2,4-Dihydroxy-6,7-diphenylpteridine	125

Eastman Kodak Company; Goodrich Chemical Company; Lederle Laboratories; Merek and Company; National Research Council; Parke Davis and Company; Protein Chemistry Department,

Sloan-Kettering Institute; Schwarz Laboratories, Inc.; Southern Research Institute.

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## 17200. A Readily Soluble form of P. B. P. for Use as a Routine Diagnostic Test.

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We reported the preparation of a purified protein antigen from *Brucella*,<sup>1</sup> made by following a modification of Seibert's method for the preparation of PPD (purified protein derivative from tuberculin). This protein substance was a fine, light brown powder of fairly constant chemical composition, not completely soluble in water but easily dissolved by the addition of a few drops of 0.1 N alkali. The solution could be neutralized with 0.1 N HCl and remained clear. This preparation was used by us, as well as by other investigators, for the study of cutaneous hypersensitiveness to *Brucella*. As workers may not have laboratory facilities, we have devised a method whereby the PBP (purified brucella protein) is put up in vial form and in just the right amount for skin testing. By adding a measured quantity of sterile saline buffered solution, the PBP is ready for use.

To prepare the PBP in this way, a weighed amount of the protein antigen is dissolved, as indicated above, to make a concentration of one mg per cc. This solution is dialyzed through cellulose tubing (Visking Corp.) in cold water for 24 hours. Any impurity that may separate is removed by centrifugation. Then 50 mg of Beta lactose per cc of the PBP solution are dissolved. The resulting liquid should be completely clear. One half cc of this solution, containing 0.5 mg of the *Brucella* protein extract, are bottled in glass vials (about 8 ml capacity) of the type ordinarily used for vaccines, and the contents dried by lyophilization. The lactose has no

deleterious effect on the PBP; does not influence the allergic reaction, and is used only to give bulk to the product after drying, since the amount deposited in each vial is infinitesimal.

For skin testing, 5 ml of the sterile diluting fluid are added to each vial, by means of a sterile syringe and needle through rubber stoppers of the vial, and shaken for 2 or 3 minutes to dissolve their contents. The diluting fluid is the same used for Seibert's PPD<sup>2</sup> and is a buffered saline liquid made from the following solutions:

$\text{KH}_2\text{PO}_4$ : 9.078 g dissolved in 1000 cc of distilled water

$\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ : 11.876 g dissolved in 1000 cc of distilled water

Two parts of the solution of the potassium salt are mixed with 8 of the sodium salt, and 0.25% phenol is added.

Each vial contains enough PBP for 50 tests. The tests are performed by injecting 0.1 ml of the diluted protein product with a tuberculin type syringe and a 26-gauge needle into the skin over the flexor surface of the forearm. The site of the injection is examined 48 hours later. Subjects who react positively show a pronounced erythema and edematous induration. Negative cases show no changes.

This preparation was tested on 25 individuals supposed to have been in contact with infected material. Two of them were veterinarians and 2, their assistants. The rest were milkers and dairy workers. Of this group 8 gave positive and 17, negative.

<sup>1</sup> Morales-Otero, P., and González, Luis M., *Proc. Soc. Exp. Biol. and Med.*, 1938, 38, 703.

<sup>2</sup> Seibert, F. B., *et al.*, *Am. Rev. Tuberculosis*, 1934, 30, 707.



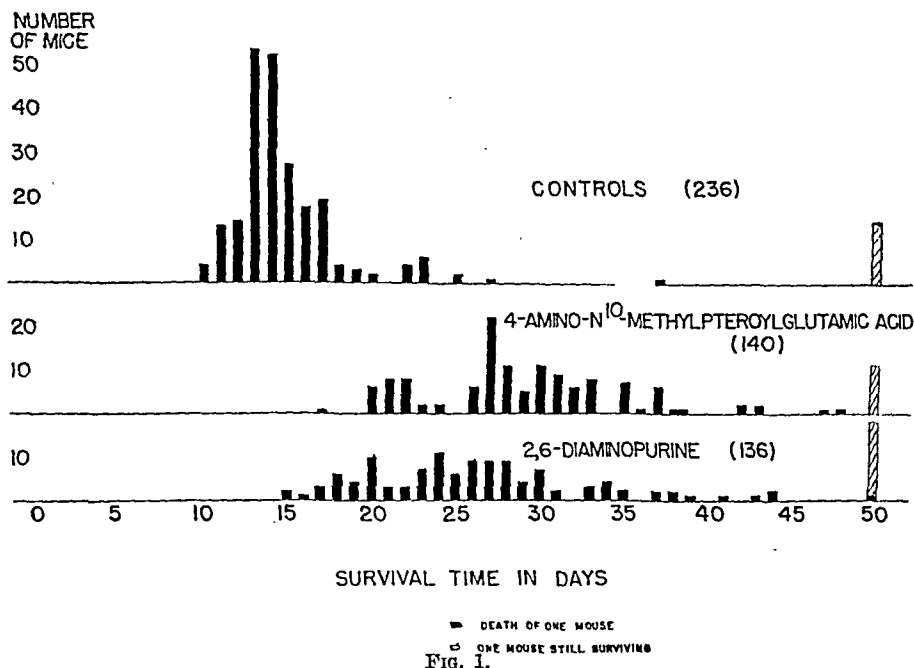


FIG. 1.

inhibition of growth of *L. casei* with PGA in the absence of purine was a property of nearly all 2,4-diamino-pyrimidines and their condensed systems.<sup>9</sup>

Despite the fact that certain simple pyrimidines<sup>10</sup> and pteridines<sup>8</sup> are antagonists of PGA in the metabolism of bacteria, it is of interest to note that all such compounds which were tested against Ak 4 leukemia were without definite effect. The addition of a para amino-benzoic acid moiety to the 2,4-diamino pteridines in 4-aminopteroic acid (2,4-diamino-6-p-carboxyanilinomethylpteridine) and in 4-amino-N<sup>10</sup>-methyl-ptericoic acid (2,4-diamino-6-N-methyl-p-carboxyanilinomethylpteridine) did not increase activity. With the exception of 2,6-diaminopurine, the 2,4-diamino configuration of the pyrimidine ring was effective only when it was a portion of a larger molecule consisting of a pteridine, a para amino-benzoic acid, and an  $\alpha$ -amino acid. 4-amino derivatives containing the  $\alpha$ -amino acids, glutamic, aspartic or threonine, pos-

sessed chemotherapeutic activity, but 4-amino-pteroyl alanine was inactive.

**Summary.** 1. Ninety compounds related to pteroylglutamic acid have been tested for chemotherapeutic effect against transmitted leukemia Ak 4 in mice.

2. Eighty-two of these compounds showed no chemotherapeutic effect by this particular technic.

3. Four showed slight to moderate effect.

4. Four compounds, 4-amino-N<sup>10</sup>-methyl-pteroylglutamic acid, 4-amino-9-methyl-pteroylglutamic acid, 4-amino-9,10-dimethyl-pteroylglutamic acid, and 2,6-diaminopurine have definite chemotherapeutic activity as demonstrated by approximately doubling the average survival time of the mice treated with these compounds.

5. The occurrence of an amino substitution in the second and fourth positions of the pyrimidine ring in all these active compounds has been noted.

We wish to acknowledge at this time the generosity of the following groups in supplying the compounds used in these studies: Dr. C. K. Cain, Department of Chemistry, Cornell University; Calco Chemical Company; Commercial Solvents;

<sup>9</sup> Hitchings, G. H., Elion, G. B., Vander Werff, H., and Falco, E. A., *J. Biol. Chem.*, 1948, **174**, 765.

<sup>10</sup> Hitchings, G. H., Falco, E. A., Sherwood, M. B., *Science*, 1945, **102**, 251.

this strain.<sup>2</sup> Of these two sites, the testis was used since it contains numerous primitive mesenchymal cells. It nicely delimits the area affected, and it does not contain or allow any contact with the type of connective tissue associated with ligaments or bone attachments. The bone extract was made according to the procedure of Annersten,<sup>5</sup> with slight modification to adapt it to the mouse. All of the long bones, the pelvis, the shoulder girdles, the calvarium and any other bones that could be readily freed from the surrounding tissue were removed from 6 or 7 mice. These bones, weighing 5 to 8 g were ground with a mortar and pestle and extracted with 15 to 20 cc of HCl-alcohol (100 cc of 95% alcohol and 5 cc of 0.1N HCl). The ground bone was placed in a glass stoppered tube. The alcohol was added and stirred. The mixture was then placed in a refrigerator at  $+1^{\circ}\text{C}$  for 48 hours and stirred frequently. It was then centrifuged. The supernatant liquid was placed in an appropriate container and evaporated to one-quarter its former volume by passing a stream of warm air over it. The extract became opalescent and a fine lipid film formed around the edges of the surface. No attempt was made to analyze the content of this extract. However, Annersten<sup>4</sup> gives a report on the analysis of his extracts of rabbit bones. The solution was next diluted with an equal volume of isotonic salt solution and injected immediately. If injections were not to be made at once, the dilution was not done until just before injection. The extract was stored in the refrigerator at  $+1^{\circ}\text{C}$ .

The extract was injected by means of a 1 cc tuberculin syringe equipped with a 25 gauge needle. The mice were anesthetized, and the left testis was exteriorized through a suprapubic midline incision. The testis was grasped by the gubernaculum, and the hypodermic needle inserted at the posterior pole of the testis, parallel to its long axis. As much extract as could be retained (about 0.05 cc) was injected. If too much solution was injected, the internal pressure caused herniation of the seminal tubules at the point of entrance of the hypodermic needle. The testis was then returned to the abdominal cavity and the incision sutured. Thirty-three male mice

were used. The animals were autopsied at 15 days, and at 3, 4, 5 and 6 weeks after the extract was injected. The testes were preserved in Bouin's fluid, sectioned in paraffin ( $7\ \mu$ ) and stained with Harris' hematoxylin and triosin.

*Observations.* In gross appearance the treated testes are somewhat less than  $2/3$  their normal volume. If the seminal tubules herniate, or some of the acid-alcohol extract leaks out, there is a tendency for the testis to adhere to surrounding tissue. Adhesion usually occurs in the open inguinal canal or in the region around the abdominal incision. The area that is affected by the extract can be readily seen in gross observation, since the tubules appear as chalky white structures showing through the tunica albuginea. The remainder of the testis is the normal pinkish gray color without the tubular nature of the testis being apparent. The extract seems to fix the tissue somewhat in about  $2/3$  of the testis (Fig. 1). At 2 weeks there are no lumina in the tubules of the treated area. The cell membranes are no longer apparent, and the coalescence of the cells produces a homogeneous light pink-staining granular mass. Apparently the nuclei of the basal layers of cells disappear, but the chromatin material of the spermatids and spermatozoa is grouped concentrically at the centers of the tubules. Nuclear membranes are not seen, yet the chromatin material is very dense and compact, without evident coalescence, and retains its hematoxylin-staining affinity. The connective tissue forming the tubule walls appears desiccated, but otherwise does not show much alteration. The interstitial tissue is composed of connective tissue, Leydig cells and occasional macrophages and lymphocytes. The cytoplasm of the connective tissue cells and Leydig cells takes the same light pink stain as does the material within the tubules. Some of the Leydig cells have the characteristic interstitial cell nuclei, while in others the nuclei are becoming pycnotic. The nuclei of the fibroblasts are in most instances pycnotic. Lymphocytes are not yet numerous, and the blood vessels show only slight evidences of future hyalinization. Macrophages are only occasionally seen.

As a further check of the specificity of this lyophilized form of PBP, 10 patients from the University Hospital, who had been diagnosed as brucellosis cases and who had previously given a positive skin test with the old form of PBP, were again tested with this readily soluble form. All gave a positive skin reaction.

For negative control group, 20 healthy subjects, selected at random from the general population and with no history of brucellosis or symptoms usually associated with this condition, were also tested; all were negative to

the lyophilized PBP.

*Summary.* A purified brucella protein, subjected to further purification and presented in a soluble lyophilized form, has been described. Weighed amounts are placed in glass vials, which, after the addition of a measured quantity of sterile saline buffered solution, produces a product ready for use. This procedure facilitates the use of the product in epidemiological investigation and as a routine diagnostic test.

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## 17201. Effects of Bone Extracts Injected into the Mouse Testis.\*

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Bone will develop from bone marrow when it is transplanted under certain conditions. This subject has recently been reviewed by Levander<sup>1</sup> and Pfeiffer.<sup>2</sup> However, these two authors differ markedly in their interpretation of how bone is formed from the marrow transplant. Levander<sup>1</sup> has reported that when bone marrow is transplanted, all of the grafted cells disintegrate and mesenchymal cells from the surrounding tissue soon migrate into the graft and are transformed into chondroblasts and osteoblasts which form cartilage and bone. Pfeiffer<sup>2</sup> agrees with Levander that the hematogenic and myelogenic cells disappear from the graft but believes that when bone forms, the marrow reticulum cells of the graft survive and these cells develop into osteoblasts just as they do in the marrow cavity when medullary bone forms there as described by Bloom, Bloom and McLean<sup>3</sup> in the pigeon.

The evidence in the literature<sup>4-7</sup> that there is an extractable osteogenic substance would tend to support Levander's hypothesis. However, it has been questioned whether such a substance exists, and evidence that injury is the primary factor in causing bone to form when bone extracts are injected has been advanced by Heinen.<sup>8</sup> Apparently mesenchymal cells transform into osteoblasts in either case. It therefore becomes important to test whether the mesenchymal cells present in the area where bone developed in marrow grafts would produce bone under the same or greater injury than resulted from grafting, plus the extract from a similar or larger piece of marrow than was grafted.

*Materials and methods.* Mice of the Strong<sup>9</sup> A strain were used in this experiment because it has been shown that bone will develop in marrow grafts in the anterior chamber of the eye and in the testes of mice of

\* This investigation was aided by grants from The Anna Fuller Fund, the National Cancer Institute (U.S.P.H.S.), and the James Hudson Brown Fund.

<sup>1</sup> Levander, G., *Acta Chir. Scand.*, 1940, **83**, 545.

<sup>2</sup> Pfeiffer, C. A., *Anat. Rec.*, 1948, **102**, 225.

<sup>3</sup> Bloom, W., Bloom, M. A., and McLean, F. C., *Anat. Rec.*, 1941, **81**, 443.

<sup>4</sup> Annersten, S., *Acta Chir. Scandinav.* (Suppl. 60), 1940, **81**, 1.

<sup>5</sup> Annersten, S., *Chirurg.*, 1941, **13**, 76.

<sup>6</sup> Bertelsen, A., *Acta Orthopaedica Scandinavica*, 1944, **15**, 139.

<sup>7</sup> Levander, G., and Willstaedt, H., *Naturc.*, 1946, **157**, 587.

<sup>8</sup> Heinen, J. H., personal communication, 1948.

<sup>9</sup> Strong, L. C., *J. Hered.*, 1936, **27**, 21.

All tissues were fixed in Bouin's fluid, sectioned at  $7\ \mu$  and stained with Harris' hematoxylin and triosin.

FIG. 1. Longitudinal section through entire testis and epididymis following injection of 0.05 cc of an acid alcoholic extract of bone into the testis. The portion to the left is the treated area and that to the right is the normal testicular tissue. Note the sharp dividing line between the infarct and the normal area.  $\times 12$ .

FIG. 2. A section of the treated area of a testis 20 days after injection of an acid alcoholic extract of bone. This section is near the normal part of the testis and shows the predominance of fibroblastic elements in the interstitial tissue of this area and the disappearance of all normal cellular structure in the seminiferous tubules, except for the darkly staining chromatin material of the spermatids and spermatocytes which is arranged concentrically in the center of the tubules.  $\times 110$ .

FIG. 3. Same testis as in Fig. 2. A section near the center of the treated area. Note the rather normal appearing Leydig cell between the 2 tubules at the extreme right and the numerous cells in the interstitial tissue, some of which may be altered Leydig cells but most of which are undoubtedly macrophages. See also altered protoplasm, lack of chromatin material and even, faintly staining appearance of all spermatogenic cells. The chromatin material of the spermatids and spermatozoa still stains very strongly, however.  $\times 220$ .

FIG. 4. A cross section of an arteriole at periphery of infarct in a testis 20 days after injection of bone extract, showing marked alteration of these vessels and presence of numerous plasma cells and lymphocytes around them. The remaining interstitial tissue appears greatly dehydrated but not otherwise much altered.  $\times 220$ .

FIG. 5. Interstitial tissue 4 weeks after injection of bone extract. Note little change in the cells. They are extremely pycnotic, and the fibers appear denser. The small blood vessels show none of the changes seen at periphery. All of tissue has been killed and fixed by the alcohol and remains in this condition with no evidence of removal of dead tissue.  $\times 220$ .

FIG. 6. The normal portion of a testis that had received acid alcoholic extract of bone 6 weeks before. The treated area of the same testis is shown in Fig. 8.  $\times 220$ .

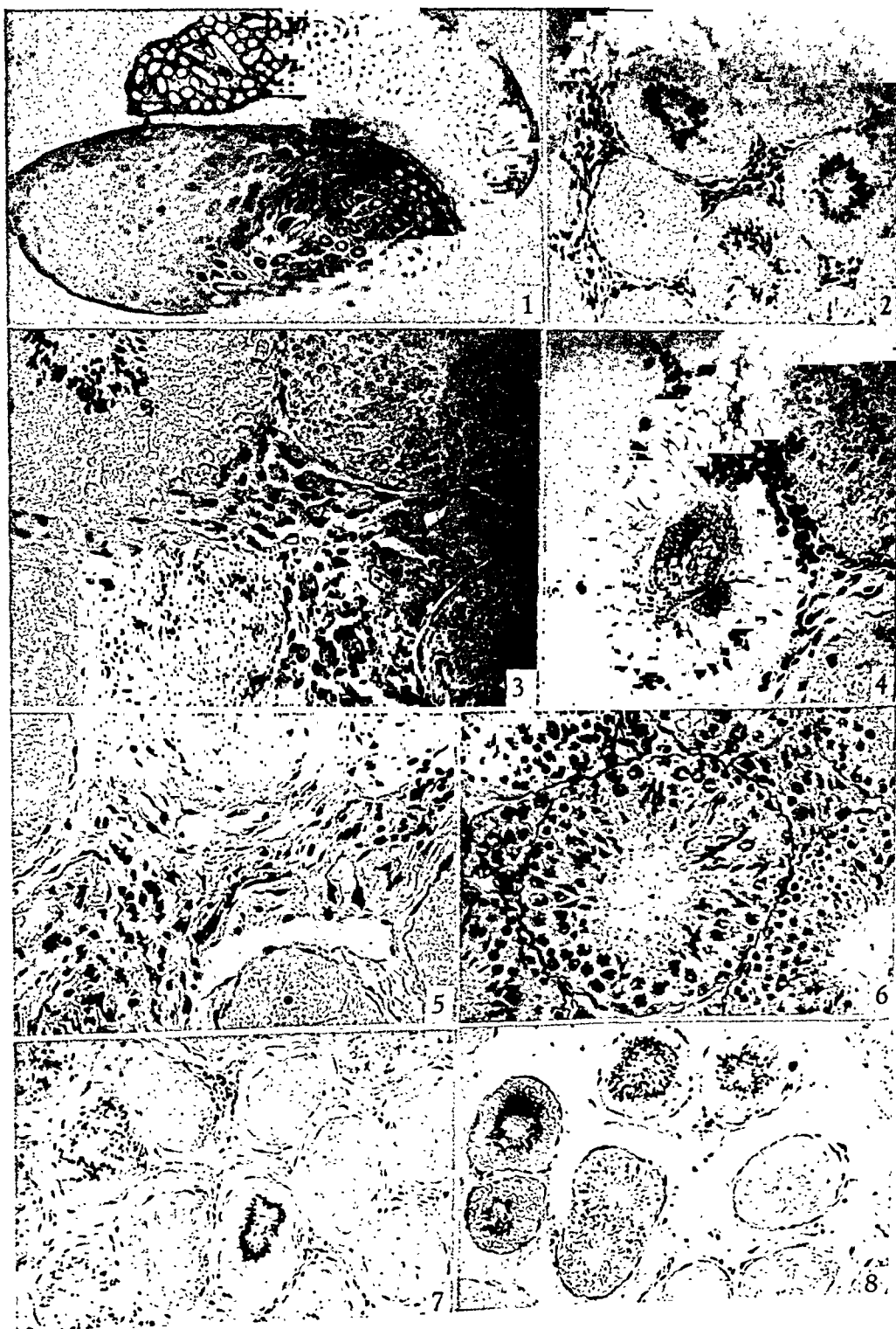
FIG. 7. Treated area of a testis 5 weeks after injection of bone extract. There is little change from earlier stages except that there is more interstitial edema. The tubules are smaller and there are possibly fewer chromatin remnants of the spermatids and spermatozoa in the tubules.  $\times 110$ .

FIG. 8. Treated area from the same testis as shown in Fig. 6. It is similar to Fig. 7 except for the greater interstitial edema. At 6 weeks there is no evidence that the killed and preserved cells are being cleared out of the area.  $\times 110$ .

At 3 weeks the contents of the seminiferous tubules are essentially the same as at 2 weeks (Fig. 2). The protoplasm is, however, somewhat more homogeneous. In the intertubular spaces there are fewer cells with definitive interstitial cell nuclei, but cells with pycnotic nuclei which might formerly have been interstitial cells of Leydig are fairly numerous (Fig. 3). They are, however, almost completely absent from the center of the treated area. The fibroblasts show little if any change from that seen at 2 weeks. A few polymorphonucleated cells are present at the periphery of the treated area. Also in this region are a few lymphocytes. The vascular condition has changed very little from that at 2 weeks. Hyalinization of the blood vessels is beginning at the periphery, and lymphocytes are becoming more numerous around these blood vessels (Fig. 4). At the dividing line between the treated and normal areas an occasional seminiferous tubule shows the characteristic clumping of the spermatocytes that occurs when the seminiferous tubules are damaged.

This type of degeneration in the tubules is never seen except at the periphery of the treated area, and is secondary to the primary damage.

By 4 weeks the tubules are further shrunken in size. The protoplasm appears to be more homogeneous. There is less chromatin material visible, and many cross sections of tubules show none at all. There is much more intertubular edema, although this varies considerably from animal to animal. The nuclei of the fibroblasts are quite pycnotic (Fig. 5), and only rarely can a cell be found which could have been an interstitial cell of Leydig, even in the peripheral zone of the affected area. There is a somewhat marked hyalinization of the blood vessels, but it is only the vessels at the periphery of the treated area that show this response. Lymphocytes are much more numerous, especially near the blood vessels, and plasma cells are now present. Macrophages are not noticeable at this time. The amount of connective tissue in the interstitial spaces is about normal, but it ap-



bone when bone marrow is transplanted, just as they have been shown to do in their normal position,<sup>3</sup> the evidence is equally convincing that bone is caused to form in many areas where marrow reticulum cells are not present. The osteoblasts and chondroblasts in the latter areas must come from the primitive mesenchymal cells. However, in spite of the obvious similarity of the primitive mesenchymal cells and the marrow reticulum cells new problems become evident. The injection of an acid alcoholic extract of bone into muscle has been reported to cause the primitive mesenchymal cells to form bone,<sup>1,4-6</sup> yet the testis of the mouse contains large numbers of primitive mesenchymal cells which failed to respond to acid alcoholic extract of bone. In the present experiment all the tissue in the treated area is killed. It represents a subtotal infarction, and apparently the damaged tissue is gradually removed by autolytic enzymes brought in by the tissue fluids which penetrate the area. No cells remain in the treated area that could form bone. If bone should form under these circumstances it would have to be from cells outside the treated area or from cells which migrate into the area by way of the blood stream or by connective tissue ingrowth from the periphery. It would not be expected that any injected osteogenic substance would remain in the area for as long as these experiments ran. Therefore, if bone should develop later, it could hardly be attributed to the injected osteogenic substance. It is also evident that the injury produced by injection of the bone extract, which Heinen<sup>2</sup> believes is responsible for bone formation under these conditions, did not cause bone to develop in the testis. It is true that we did not pretreat the area with 40% alcohol as Annersten<sup>4,5</sup> did, but with the limited area within the testis and the extreme damage to the tissue, it was felt that the effect of alcohol pretreatment would only be to further limit the amount of extract that could be retained. Moreover, Bertelsen<sup>6</sup> has claimed that this pretreatment is not necessary.

The fact that the presence of alcoholic ex-

tract of bone and the considerable injury produced by its presence has not caused bone to form in the mouse testis, while marrow transplants readily produce bone there, suggests that in the mouse the primitive mesenchymal cells of the testis are not easily stimulated to form bone. Therefore, the mouse testis is not a good place to test either whether any extractable osteogenic substance exists, as postulated by Annersten<sup>4,5</sup> and Bertelsen,<sup>6</sup> or whether injury to the tissue brought about by the extract is the cause of the response when bone formation follows the injection of alcoholic extracts into muscle.<sup>2</sup> The rabbit, which has been used in the experiments testing the presence or absence of an osteogenic factor, on the other hand is notorious for the ease with which ossification occurs following injury, a fact which has been becoming increasingly evident since the experimental production of ectopic bone in the rabbit by Sacerdotti and Frattin.<sup>15</sup> It seems probable that the present failure to obtain bone formation is related to the use of the mouse as an experimental animal and the testis as the injection site. It was not thought feasible to test the bone extract on the muscle of the mouse since it would be unlikely that appreciably more extract could be injected here than into the testis without affecting the connective tissue involved in tendon or muscle insertion.

*Summary.* An acid alcoholic extract of bone did not cause the development of bone when it was injected into the testes of 33 male mice of the Strong A strain. It caused extreme damage to about 2/3 of the testis, producing a subtotal infarction of the area. Since fragments of bone marrow produce bone when transplanted to the testis, whereas extracts from much larger amounts of marrow and bone do not cause the development of bone, it is concluded that the marrow reticulum cells survive grafting and produce the bone.

<sup>15</sup> Sacerdotti, C., and Frattin, G., *Firchows Arch. f. path. Anat.*, 1902, 108, 431.

pears dehydrated, and the large empty spaces due to further shrinkage of the tubules make it appear less abundant.

From the fourth to the sixth week the changes already described become more extreme (Fig. 7 and 8). There is further shrinkage of the tubules and disappearance of chromatin material, although certain tubules resemble those at 2 or 3 weeks, except for the smaller size. Usually only connective tissue which has been coagulated by the injected alcohol is seen in the intertubular spaces. The fibers often show evidence of disintegration. The spaces between the tubules, and between the tubules and connective tissue become greater. Apparently the shrinkage of the tubules is much greater than that of the connective tissue. In the central area only an occasional cell that may be a degenerating interstitial cell or a macrophage is seen. If these are living macrophages, there is no evidence of their phagocytic activity.

The vascular changes seen in the treated testes seem to be associated with the peripheral area, and the presence of lymphocytes and plasma cells is limited primarily to this region. Presumably the lymphocytes come in from the blood stream, but the plasma cells may possibly be formed in the area. There is no evidence whether the plasma cells arise from lymphocytes or not. They are, however, limited to the same areas. There seems to be no evidence of undamaged fibroblasts or primitive mesenchymal cells in the treated area. The vascular and lymphocyte response in the peripheral region occurs whether it is adjacent to normal seminiferous tubules or to the tunica albuginea. The tubules in the area outside of that exposed to the extract are essentially normal (Fig. 6) except for a slightly cryptorchid appearance which may be due to the fact that the testis is only about 2/3 its normal size and probably has resided in the abdominal cavity most of the time. The interstitial tissue and Leydig cells appear to be increased somewhat in this area. This is probably due to the reduced tubular volume.

**Discussion.** The fact that bone did not develop in the mouse testis when acid alcoholic extract of bone was injected, even though the amount of extract injected represented a

volume of marrow many times that which was transplanted to the mouse testis and which formed bone,<sup>2</sup> strongly suggests that the marrow graft contributes something besides a possible extractable osteogenic substance, presumably the growing marrow reticulum cells, which it seems probable become osteoblasts and form bone.

In the literature on the transplantation of bone marrow the following facts are evident: 1. Autoplastic transplants of bone marrow are fairly successful in forming bone.<sup>1,10-14</sup> 2. Homoplastic transplants are seldom successful<sup>10,11,15,16</sup> and then only in young animals, and with large amounts of marrow.<sup>1,17</sup> 3. In inbred strains of mice implantation of tiny fragments of marrow cause the development of bone.<sup>2</sup> 4. The degree of resistance to grafting which is present in the grafting site is of extreme importance; where resistance is high, bone has not formed when marrow is transplanted.<sup>2</sup> All of these conditions are related to the ability of grafted cells to grow in their new environment. It seems, therefore, that bone develops in marrow transplants only under conditions that are favorable for transplantation. Since all workers agree that the myelogenic and hematogenic cells do not survive grafting, it must be that the marrow reticulum cells do survive as suggested by Pfeiffer<sup>2</sup> and are the cells which Levander<sup>1</sup> believes are mesenchymal cells which have migrated into the area.

While it seems quite clear from the above evidence that marrow reticulum cells form

<sup>10</sup> Baikow, A., *Centralbl. f. d. Med. Wiss.*, 1870, 8, 371.

<sup>11</sup> Bruns, P., *Arch. f. klin. Chir.*, 1881, 26, 661.

<sup>12</sup> Bull, Ch. R., *Experimentelle Studien über Knochen transplantation und Knochenregeneration*, 1928, J. Dybwad, Oslo.

<sup>13</sup> Chiari, O. M., *Münch. Med. Woch.*, 1912, 59, 2502.

<sup>14</sup> Miyachi, K., *Arch. f. klin. Chir.*, 1914, 106, 273.

<sup>15</sup> Ollier, L., *Traité expérimentale et clinique de la régénération des os et de la production artificielle du tissu osseux*. T. I, partie expérimentale, 1867, Victor Masson et Fils, Paris.

<sup>16</sup> Maas, H., *Arch. f. klin. Chir.*, 1887, 20, 708.

<sup>17</sup> Goujon, E., *J. de L'Anat. et de La Physiol.*, 1869, 6, 399.

TABLE I.  
Incidence of Lesions in the Kidney and Blood Pressure of 100 Rats Following Unilateral Nephrectomy.

Mean blood pressure in mm Hg	Up to 120	120 to 130	130 to 140	140 to 150	Over 150
No. of rats	8	15	29	28	20
Incidence of lesions in ablated kidney	0	+	++	+++	++++

TABLE II.  
Effect of Various Diets on the Survival of Rats Following Bilateral Nephrectomy.

No. of rats	Diet	Survival in days	
		Range	Avg
20	Regular	2.0 to 4.5	3.0 $\pm$ 0.4*
20	Dialyzed (sodium and potassium "free")	3.2 to 6.0	4.5 $\pm$ 0.4
20	"Protein-free" diet (glucose, starch, lard)	2.9 to 5.5	4.6 $\pm$ 0.5
20	Dialyzed food plus 2% NaCl	2.8 to 5.0	4.3 $\pm$ 0.5
20	" " " " KCl	2.5 to 4.5	3.7 $\pm$ 0.3

\* Standard deviation.

was in each case found to be elevated 30 to 50 mm of mercury over the highest level attained prior to the removal of the second kidney. Bilateral nephrectomy in the rat, as in the dog,<sup>6</sup> thus results in elevation of the blood pressure to hypertensive levels if the animals survive for 5 days or more in good condition.

*Microscopic studies of the right kidneys.* The structural deviations from normal observed microscopically in the right kidneys of the rats whose blood pressure levels are recorded in Table I could be grouped as absent, minimal, and slight. There was a conspicuous absence of any acute or chronic inflammatory change involving the glomeruli, tubules, renal pelvis, stroma or blood vessels. The minimal and slight changes observed consisted of an increase in the connective tissue stroma of occasional glomeruli and hyaline change of some glomeruli, of varying degrees or complete. Glomeruli with such changes were few and only in occasional kidneys were they associated with changes in the tubules. Comparison of the incidence of these structural changes in the kidneys with the blood pressures of the animals from which they were removed indicated with some degree of certainty that glomeruli with hyaline change were more numerous and that the associated tubular changes were conspicuous in those rats whose blood pressures were in

the hypertensive range. The structural changes were absent or minimal in the kidneys of animals, the blood pressures of which were not affected by nephrectomy.

*Survival following removal of the second kidney.* The survival times of the rats following removal of their second kidney are recorded in Table II. Removal by dialysis of the salts present in the regular diet or the use of a salt-free, protein-free diet increased the average survival times from 3 to 4.5 days. That this prolongation in the survival time is due chiefly to the removal of potassium salts from the diet is shown by the fact that the addition of sodium chloride to the dialyzed diet did not affect appreciably the period of survival, whereas the addition of potassium chloride reduced it to a period comparable to that observed with the regular diet. Complete abstinence from food or the administration of 0.9% saline in lieu of drinking water also reduced the survival period markedly. The longest survival observed under optimal conditions was 6 days. Comparable results have been reported for the dog.<sup>6</sup> The urea content of the blood of 18 of the bilaterally nephrectomized rats just prior to or at death varied from 500 to 1090 mg per 100 cc and averaged 748 mg %. These values are appreciably higher than those reported by

<sup>6</sup> Grollman, A., Vanatta, J., and Muirhead, E. E., *Am. J. Physiol.*, 1949, **157**, 21.



## 17202. Renal Lesions in Chronic Hypertension Induced by Unilateral Nephrectomy in the Rat.\*

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Chronic experimental hypertension induced in the rat resembles that observed in other species with the exception that the incidence of hypertension following operative procedures on one kidney or unilateral nephrectomy is greater than in other species. In the dog, for example, chronic hypertension ensues rarely following application of a clamp to the renal artery, removal of one kidney, or other manipulations, if the opposite kidney be left intact.<sup>1-3</sup> In the rat, on the other hand, approximately 20% of animals develop hypertension following application of a figure-of-eight ligature to one kidney or unilateral nephrectomy.<sup>4</sup> The present study was undertaken to discover the factors which determine whether or not a given animal will develop hypertension following unilateral operation in order to elucidate further the role of the kidney in the pathogenesis of this disorder. The effect of unilateral nephrectomy on the weight of the remaining kidney and the changes produced in this kidney by hypertension; the changes produced in the heart by removal of the remaining kidney; and the influence of various types of diet on the survival period of the bilaterally nephrectomized rat were also studied.

**Methods.** Healthy piebald rats (Evans-McCollum strain), one to 2 years old, were selected from our breeding colony. The blood pressures were determined daily for a period of 10 days by the plethysmographic method.<sup>5</sup>

\*Supported by a grant from the Medical Insurance Fund.

<sup>1</sup> Grollman, A., Factors Regulating Blood Pressure, Trans. Second Conference, Macy Foundation, New York, 1948.

<sup>2</sup> Goldblatt, H., *Physiol. Rev.*, 1947, **27**, 120.

<sup>3</sup> Ogden, E., Tripp, E., and Constant, G., *Fed. Proc.*, 1949, **8**, 120.

<sup>4</sup> Grollman, A., Harrison, T. R., and Williams, J. R., Jr., *Am. J. Physiol.*, 1943, **139**, 293.

Under ether anesthesia, the right kidney was removed through a lumbar incision from 110 animals, the blood pressures of which ranged between 110 and 120 mm of mercury. Subsequently, blood pressure determinations were recorded 3 times weekly. The left kidney was then removed and the nephrectomized animals were subjected to various dietary regimes to determine the effects of these diets on the survival period. When the rat died the heart was removed. The weights of each kidney and of the heart were recorded after fixation in 10% formalin. A cross section of the central portion and a longitudinal section of either the distal or the proximal portion of each kidney and a longitudinal or transverse section including both ventricles of the heart were imbedded in paraffin, cut, and stained with hematoxylin and eosin.

**Results.** *Effect on the blood pressure of unilateral nephrectomy.* The mean blood pressure levels of 100 rats, 3 months following removal of the right kidney, are recorded in Table I. The figures indicate that 23% of the animals remained normotensive or manifested no greater increase in blood pressure over the preoperative level than the limits of experimental error ( $\pm 10$  mm of mercury). The largest group, 57%, assumed an intermediate position with blood pressures up to 150 mm of mercury, which are definitely above normal. The remaining 20% of the rats developed hypertension of notable degree with blood pressures above 150 mm of mercury. These are essentially the same effects previously found to follow the application of a figure-of-eight ligature to one kidney, the other kidney being left intact. Following the removal of the second kidney, the blood pressure in those animals which survived to the fifth and sixth days

<sup>5</sup> Williams, J. R., Jr., Harrison, T. R., and Grollman, A., *J. Clin. Invest.*, 1939, **18**, 373.

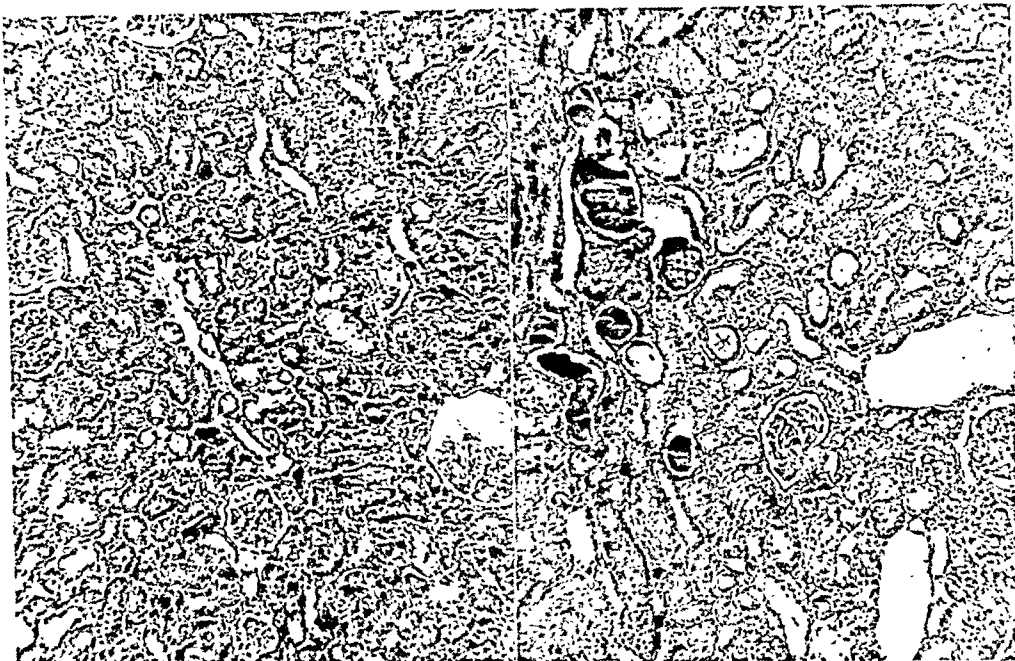


Fig. 1.

Microscopic changes in glomeruli and tubules of the right kidney (left) removal of which was followed by permanent elevation of blood pressure to 160 mm Hg. More extensive changes are seen in the left kidney (right) of the same rat (R-64-48) removed 10 weeks later.  $\times 60$ .

pertension results from the elaboration of some pressor agent unless one makes the unlikely assumption that removal of one kidney puts such a strain on the other that it excretes a pressor agent.<sup>12</sup> When both kidneys are unimpaired and one kidney is removed, the other may compensate for the removed kidney and no hypertension results. On the other hand, when both kidneys are slightly impaired and one kidney is removed, an elevation in blood pressure ensues. The fact that unilateral nephrectomy in the rat more frequently results in hypertension than the same procedure does in the rabbit<sup>13</sup> or in the dog<sup>14</sup> suggests that the rat has less reserve of kidney function than do these other experimental animals. Microscopic examination of presumably normal kidneys supports this hypothesis. However, the lesions present in the rat are not, as commonly stated, a result of preexistent pyelonephritis. They resemble

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<sup>15</sup> Halpert, B., and Grollman, A., *Arch. Path.*, 1947, **43**, 559.

<sup>16</sup> Halpert, B., and Grollman, A., *Proc. Soc. Exp. Biol. and Med.*, 1946, **62**, 273.

<sup>17</sup> Heller, H., and Dieker, S. E., *Proc. Roy. Soc. Med.*, 1947, **40**, 351; Dessau, F. I., and Oleson, J. J., *Proc. Roy. Soc. Exp. Biol. and Med.*, 1947, **64**, 278.

<sup>18</sup> Hartercroft, W. S., and Best, C. H., *Brit. Med. J.*, 1949, **1**, 423.

<sup>12</sup> Goldblatt, H., *The Renal Origin of Hypertension*, Chas. C. Thomas, Springfield, 1948.

<sup>13</sup> Grollman, A., *Am. J. Physiol.*, 1942, **142**, 666.

<sup>14</sup> Grollman, A., *Am. J. Physiol.*, 1946, **147**, 647.

TABLE III.  
Relation of Weight of Kidneys and Heart to Ultimate Blood Pressure.

Ultimate mean blood pressure in mm Hg	Up to 120	120 to 130	130 to 140	140 to 150	Over 150
	Avg wt in g per 100 g of body wt				
Right kidney	0.46	0.46	0.48	0.50	0.49
Left "	0.46	0.48	0.50	0.53	0.55
Heart	0.31	0.33	0.34	0.36	0.48

others,<sup>7-8</sup> and reflect the much longer survival period of our animals which are comparable to those reported by Rosenkrantz,<sup>9</sup> who administered aluminum hydroxide to prolong the life of his rats. The longer survival of our animals on a regular diet, we attribute to the fact that removal of the second kidney was accomplished with a minimum of trauma.

*Studies on the remaining kidney and heart.* The average weight in grams of each kidney and heart calculated per 100 g of body weight are recorded in Table III. The weights of the right kidneys were essentially the same in all animals regardless of the ultimate blood pressure attained following unilateral nephrectomy. The weights of the left kidneys, on the other hand, were increased in proportion with the elevation of blood pressure. It will be noted that removal of kidneys which on microscopic examination were normal resulted in no increase in weight of the remaining kidney whereas the kidneys of animals manifesting microscopic changes underwent maximum enlargement. There was thus no correlation between the weight of the first kidney removed and the ultimate blood pressure but a definite correlation between that of the remaining kidney and the blood pressure. The weights of the hearts increased proportionately to the elevation of blood pressure. This hypertrophy is not reflected exactly by the data of Table III since some loss of substance was likely to occur during the survival following removal of the second kidney. Microscopic studies of the left kidneys disclosed no striking differences from the right kidneys of the same rats. Occa-

sional glomeruli seemed larger and the tubules perhaps more spacious. This might account for the slight increase in weight of the kidneys. Changes also occurred in the left kidneys of those rats in which lesion of the right kidneys were minimal or slight. In these, glomeruli with hyaline change were more numerous and the associated tubules were dilated by a homogeneous, bright pink material and were lined by flat cells. These changes were marked only in those rats which developed hypertension. Typical appearances of the kidneys are shown in Fig. 1. Microscopic studies of the hearts disclosed minimal or slight hypertrophy of the left ventricle. The increase and variation in size of the myocardial fibers were most marked in the hypertensive rats. Acute changes were superimposed in all of the hearts which presumably occurred during the period following removal of the second kidney. They consisted of varying degrees of dilatation of the capillaries with focal areas of extravasation of blood and obliteration of the striations of the myocardial fibers to a degree of waxy change. These changes are comparable to those observed in the nephrectomized dog.<sup>10</sup> No acute or chronic inflammatory lesions were noted in the stroma nor were there any obvious changes in the blood vessels.

*Discussion.* The results of the present study are compatible with the view that the maintenance of a normal blood pressure is dependent on the presence in the organism of an adequate renal function.<sup>11</sup> It is impossible, on the other hand, to harmonize the present observations with the view that hy-

<sup>7</sup> Durlacher, S. H., and Darrow, D. C., *Am. J. Physiol.*, 1942, **136**, 577.

<sup>8</sup> Bondy, P. K., and Engel, F. L., *Proc. Soc. Exp. Biol. and Med.*, 1947, **66**, 104.

<sup>9</sup> Rosenkrantz, J. A., *Proc. Soc. Exp. Biol. and Med.*, 1946, **63**, 155.

<sup>10</sup> Muirhead, E. E., Vanatta, J., and Grollman, A., *Arch. Pathol.*, in press.

<sup>11</sup> Grollman, A., *Special Publications N. Y. Acad. Sci.*, 1946, **3**, 99; Factors Regulating Blood Pressure, First Conference, Macy Foundation, New York, 1947.

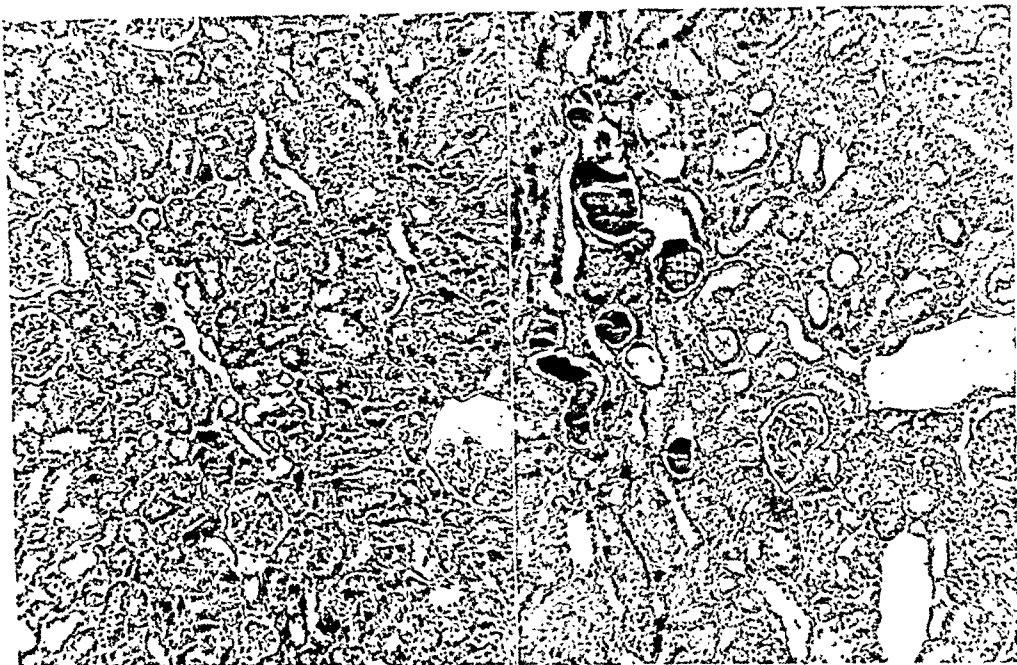


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Microscopic changes in glomeruli and tubules of the right kidney (left) removal of which was followed by permanent elevation of blood pressure to 160 mm Hg. More extensive changes are seen in the left kidney (right) of the same rat (R-64-48) removed 10 weeks later.  $\times 60$ .

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<sup>17</sup> Heller, H., and Dicker, S. E., *Proc. Roy. Soc. Med.*, 1947, **40**, 351; Dessau, F. I., and Oleson, J. J., *Proc. Roy. Soc. Exp. Biol. and Med.*, 1947, **64**, 278.

<sup>18</sup> Harteroft, W. S., and Best, C. H., *Brit. Med. J.*, 1949, **1**, 423.

<sup>12</sup> Goldblatt, H., *The Renal Origin of Hypertension*, Chas. C. Thomas, Springfield, 1948.

<sup>13</sup> Grollman, A., *Am. J. Physiol.*, 1942, **142**, 666.

<sup>14</sup> Grollman, A., *Am. J. Physiol.*, 1946, **147**, 647.

incidence of about 20% of hypertension, whereas those maintained on adequate diets rarely developed the disorder spontaneously. The existence of lesions comparable to those observed would account for the rises in blood pressure observed in the rabbit and dog following unilateral nephrectomy.<sup>6,13,14</sup>

*Summary.* The incidence of hypertension following unilateral nephrectomy is recorded in 100 rats. The development of hypertension was shown to be dependent on the status of the kidneys at the time of removal of the first kidney. If lesions are present, hypertension develops; if absent or minimal, the animals remain normotensive. The bearing of these observations on current theories as

to the pathogenesis of experimental hypertension are discussed.

The effects of unilateral nephrectomy on the weight of the heart and remaining kidney and the influence of various types of diet on the survival period following removal of the remaining kidney were also determined. Enlargement of the heart as a result of hypertrophy, as well as of the remaining kidney, may be correlated with the degree of hypertension induced by unilateral nephrectomy. Removal of potassium from the diet markedly prolongs the survival period following bilateral nephrectomy.

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### 17203. Limiting Essential Amino Acids of Some Legume Seeds.

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Pulses are one of the most important sources of vegetable proteins in human diets and knowledge of their amino acid composition is of interest especially in countries where there is insufficient consumption of animal products. The present experiments are part of a study on the nutritional problem in Venezuela, where legumes play a very important role in human nutrition.

The experiments were performed with young rats of the Sprague-Dawley strain. Each group consisted of 2 male and 2 female animals, housed individually in screen-bottomed cages. In most cases the animals were put on the experimental diet at the age of 3 weeks and weighed about 40 g each; in some cases animals weighing about 100 g were used. The diets used were similar to those described by Russell *et al.*<sup>1</sup> and contained 10% of protein and 4-5% of fat, with the exception of the soybean diets which contained 7% of fat. Food and water were given *ad libitum*. All animals received 3 drops of liver extract 3 times weekly by dropper;

this amount was sufficient to promote maximum growth on a soybean-corn diet when tested by a method previously described.<sup>2</sup> Each group received the unsupplemented diet for 3 weeks, immediately followed by the supplemented diet for the same time. In some cases the diets were given for 10 periods only. Animals were weighed every 5 days and food consumption was recorded. Digestibility was determined for all the legumes studied. Since good correlation between growth and protein efficiency was observed in accordance with the results of other authors,<sup>3</sup> only weight changes are reported.

The legume seeds were obtained from the Genetical Division of the Ministry of Agriculture in Maracay or were purchased from the local market. All seeds were ground and autoclaved at 10 lb. pressure for 20 minutes with the exception of the kidney and hyacinth beans, which were soaked in water over night, autoclaved for 30 minutes at 10 lb. pressure,

<sup>2</sup> Jaffé, W. G., and Elvehjem, C. A., *J. Biol. Chem.*, 1947, 169, 287.

<sup>3</sup> Hegsted, D. M., and Worcester, J., *J. Nutr.*, 1947, 33, 685.

<sup>1</sup> Russell, W. C., Taylor, M. W., Mehrhof, T. G., and Hirsch, R. R., *J. Nutr.*, 1946, 34, 491.

TABLE I.

Weight Changes of Rats Receiving Diets Containing Legumes as Only Source of Protein, With, or Without, Methionine Supplementation.

Legume	Scientific name	No. of samples studied	Wt change/day/animal on basal diet	Wt change/day/animal on diet supplemented with 0.3% methionine
Black kidney beans	<i>Phaseolus vulgaris</i>	4	0.0	3.1
Red " "	" "	3	-0.1	2.5
White " "	" "	1	0.9	2.6
Adzuki " "	" <i>angularis</i>	1	1.0	1.8
Mung " "	" <i>aureus</i>	1	0.5	2.3
Cow peas	<i>Vigna sinensis</i>	4	0.3	1.5
Soy beans	<i>Glycine soja</i>	3	1.3	2.5
Split peas	<i>Pisum sativum</i>	2	-0.1	0.9
Dried green peas	" "	1	0.2	1.7
Lentils	<i>Lens esculenta</i>	2	-0.1	0.5
Chick peas	<i>Cicer arietinum</i>	2	1.3	2.9
Hyacinth beans	<i>Dolichos lablab</i>	2	-0.3	4.4
Pigeon peas	<i>Cajanus indicus</i>	4	0.2	0.3*
Casein		1	2.0	4.2

\* Supplemented with 0.075% tryptophan and 0.3% methionine

0.2

3.1

then dried and ground. This procedure was adapted because these seeds contained a toxic principle in the raw stage which has been found not to disappear completely after autoclaving the dry ground material.<sup>4</sup>

With the exception of soybeans and chick peas, none of the legumes studied promoted more than very slight growth in rats if present in the diet in amounts corresponding to 10% of protein. Dried and pigeon peas did not cause good growth even when fed together with methionine. Diets containing kidney beans or hyacinth beans supplemented with methionine promoted as good or better growth than did soybeans supplemented with methionine and comparable with casein.

The data show that under the experimental conditions, methionine was a limiting indispensable amino acid in all the samples. In

the cases where previous studies exist, our results are in accordance with published data.<sup>1,5-7</sup> The results obtained with pigeon peas show that the value of the proteins for growing rats is limited by both methionine and tryptophan, as supplementation with one of these alone caused no growth response, while a combination of both resulted in enhanced weight increase.

**Summary.** The essential amino acid of legume seeds limiting growth of young rats was methionine in all cases studied, with the exception of pigeon peas, in which methionine and tryptophan were both limiting under the experimental conditions.

<sup>5</sup> Block, R. J., and Michell, H. H., *Nutr. Abstr. and Rev.*, 1946, **16**, 249.

<sup>6</sup> Richardson, L. R., *J. Nutr.*, 1948, **30**, 451.

<sup>7</sup> Belton, W. E., and Hoover, C. A., *J. Biol. Chem.*, 1948, **175**, 377.

<sup>4</sup> Jaffé, W. G., *Experientia*, 1949, **5**, 81.

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# 17204. Construction and Application of Shielded Silver Electrodes with Mercury Leads Designed for Prolonged Stimulation Experiments.\*

W. G. KUBICEK, F. J. KOTTKE, R. B. HARVEY AND D. J. LAKER.

*From the Departments of Physical Medicine and Physiology, University of Minnesota Medical School, Minneapolis, Minn.*

Prolonged electrical stimulation of the nervous system of conscious animals requires shielded electrodes which will not injure the nerves or cause undue local tissue reaction. The leads connecting the electrodes to the external electrical circuit must be capable of withstanding the almost continuous motion of a normal animal for periods of several months. The purpose of this report is to describe in detail the construction of electrodes and leads which have fulfilled the above requirements. The production of arterial hypertension by renal artery-nerve or splanchnic nerve stimulation with similar electrodes has been reported.<sup>1-4</sup>

The electrodes are constructed of No. 18 fine silver wire and are imbedded in a molded lucite block (Fig. 1). The silver electrode wire is exposed on the inner surface of the lucite block to provide direct contact with the nerve. The obturator occludes the slot in the block after the nerve is in place. The hollow stainless steel terminal is filled with mercury and connected to a mercury-filled, double lumen rubber tube which provides electrical contact to the external electrical circuits.

A double lumen latex tube joined in a Y at the stainless steel terminal provides a means of flushing and cleaning the mercury columns whenever necessary. 1N HCl, followed by water, alcohol and air, is injected into one tube, flowing through that tube, through the hole in the stainless steel ter-

minal and out through the second tube. Then this system is refilled with clean mercury.

*Construction of electrodes.* The electrode blocks are molded from powdered lucite<sup>†</sup> at a temperature of 175°C and a pressure of approximately 1000 lbs./in.<sup>2</sup> (Fig. 2).

Step 1: (Fig. 2, photo 1). The silver wire (b) is wrapped around the mandrel (c) and soldered to the stainless steel terminal (a). Soldering flux designed for stainless steel facilitates this step.<sup>‡</sup>

Step 2: The plate (h) (Fig. 2, photo 4) is placed in the bottom of the holder (j). The assembly in (1) is then placed in the holder (j) with the flat portion of the mandrel (c) inserted into the slot in the plate (h). The screw (k) is then tightened. The guide (g) (Fig. 2, photo 5) is now placed in the holder (j) and the cylinder (d) (photo 2) is attached to the holder (j). The entire assembly is filled with powdered lucite to about  $\frac{1}{8}$  inch from the top of the cylinder. Tapping the assembly will insure complete filling. The piston (e) (photo 2) is inserted into the cylinder and the complete unit is placed in the press. The springs are tightened until they are nearly closed.<sup>§</sup> The press and molding assembly is placed in an oven at 175°C. After 20 minutes the springs are again tightened and the entire apparatus is returned to the oven for 15 minutes. After the final baking the mold is allowed to cool slowly to room temperature before releasing the spring tension. When the cylinder (d) (Fig. 2,

\* Supported by a grant from the Life Insurance Medical Research Fund.

1 Kottke, F. J., Kubicek, W. G., and Visscher, M. B., *Am. J. Physiol.*, 1945, **145**, 38.

2 Kubicek, W. G., and Kottke, F. J., *Fed. Proc.*, 1946, **5**, No. 1.

3 Kottke, F. J., and Kubicek, W. G., *Fed. Proc.*, 1947, **6**, No. 1.

4 Kubicek, W. G., and Kottke, F. J., *Fed. Proc.*, 1947, **6**, No. 1.

† E. I. DuPont de Nemours Co., Plastic Dept., Arlington, N. J. Crystal, Color H7500, Comp. HIG 22, Gran Fine No. H18876.

‡ The Lloyd S. Johnson Co., 2241 Indiana Ave., Chicago, Ill. Stainless steel soldering flux.

§ Eiler Spring Co., 637 Sexton Building, Minneapolis, Minn. .125" wire coil compression springs, 10 turns 2 inches in length,  $\frac{1}{2}$ " inside diameter, approximately 90 lbs. to compress spring completely.

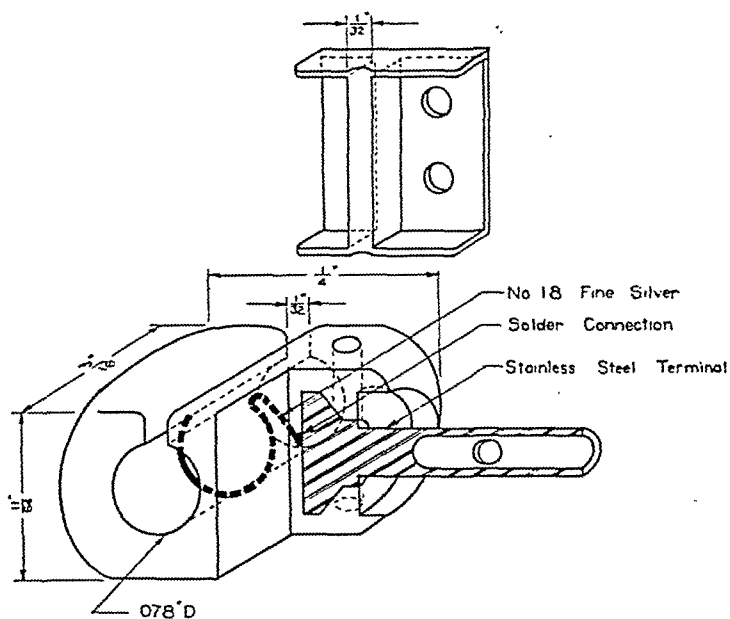


FIG. 1.  
Pictorial diagram of lucite electrode block and obturator.

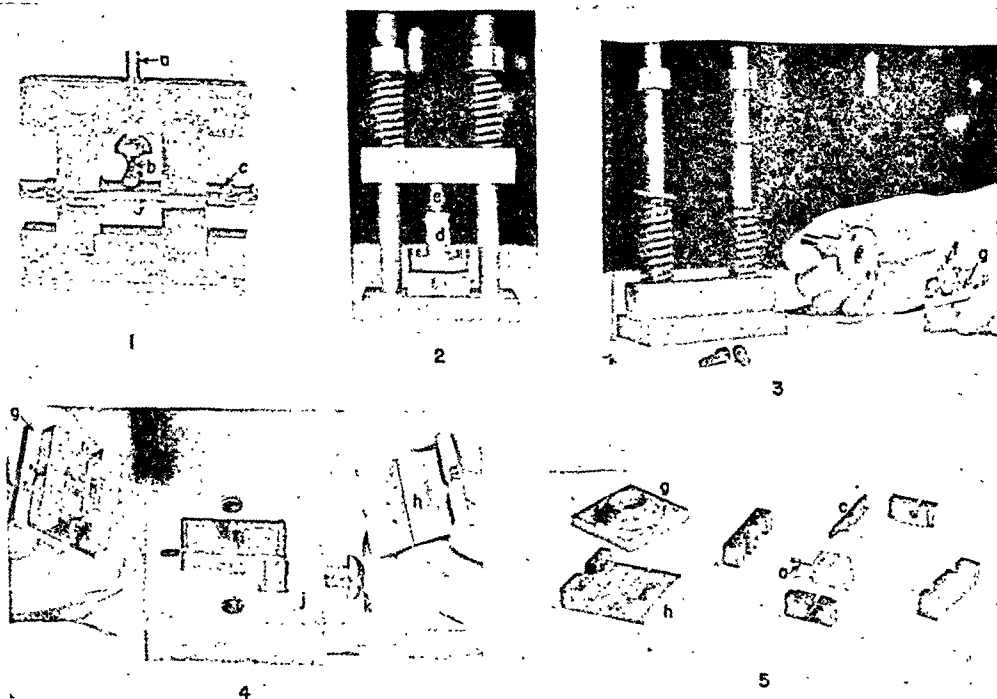


FIG. 2.  
A composite photograph of the various steps in the molding process.



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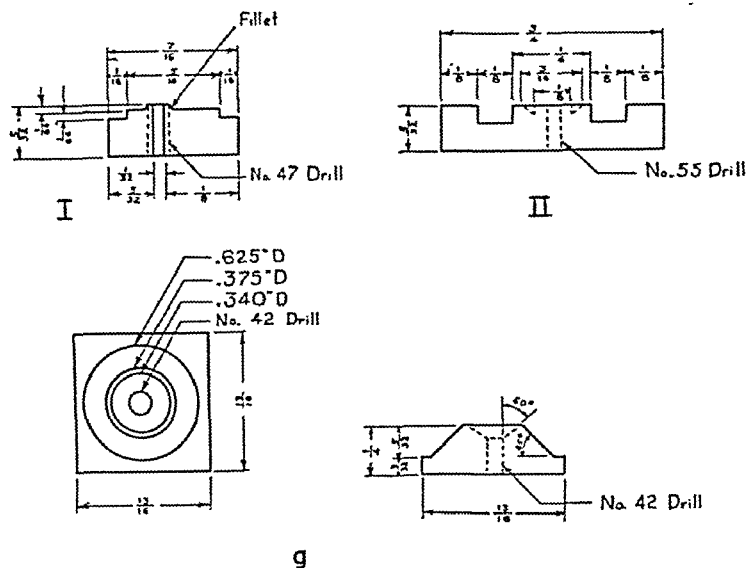


FIG. 4.  
Detail drawing of mold parts I and II of Fig. 3 and guide (g) (Fig. 2) (photo 5).

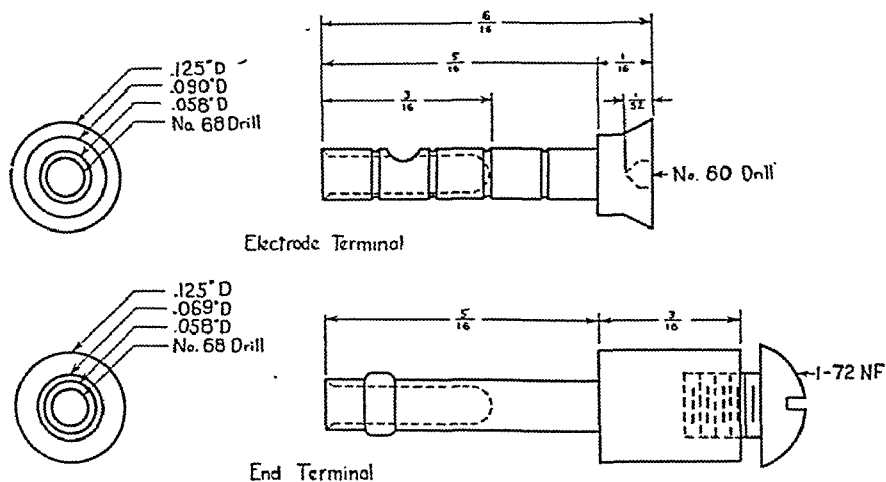


FIG. 6.  
Detail drawing of electrode terminal and end terminal.

rounding off the corners and sharp edges the lucite is polished and the holes drilled for the ligatures (Fig. 1). Lubricating the drill with kerosene will facilitate drilling lucite. During the molding process a thin film of lucite forms over the silver electrode. The film is removed with a dental grinder.

Details of the molding apparatus are presented in Fig. 3-7.

**Step 4: Construction of small double lumen latex tubes.** Stainless steel wires are joined with Wood's metal (Fig. 8). The wires are then dipped into a clay or mica release medium,<sup>4</sup> then into a coagulant,<sup>5</sup> and finally into latex.<sup>5</sup> The wall thickness of the

‡ American Anode Company, Akron, Ohio. Clay or mica release medium, No. 545 coagulant 60% centrifuged latex (keep latex at pH 9).

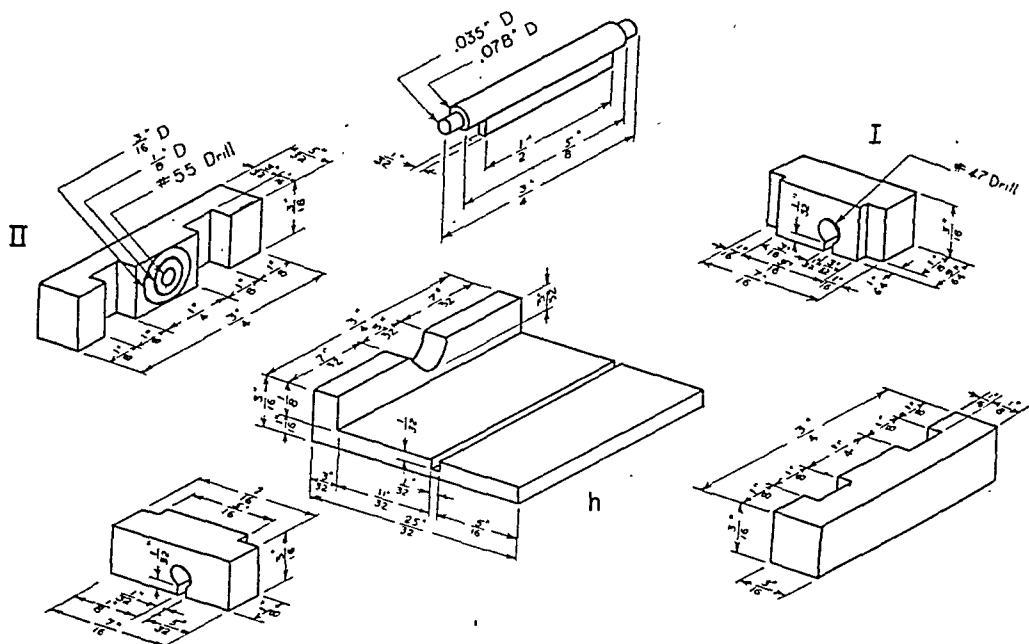


FIG. 3.  
Assembly drawing of electrode mold.

photo 3) is removed, a lucite bar (f) is found attached to the bottom of the electrode block. This bar is twisted off and the guide (g) removed. The unit in photo 1 is placed astraddle a hole in a piece of wood in such a way as to allow the mandrel (c) to be driven out with a punch.

Step 3: Obturators (Fig. 1) are molded from powdered lucite by forcing molten lucite into a plaster cast of a wax impression of the slot in the electrode block. To form a wax impression, the electrode block is first painted with an aqueous soap solution. Inlay casting wax is softened over a flame and forced into the slot with pressure applied with the thumb. Molten wax is then added to strengthen the impression. The excess wax is trimmed off leaving small tabs at the corners to act as guides on the obturator (Fig. 1). After cooling under tap water a No. 48 drill is passed through the electrode block to remove the excess wax protruding into the lumen of the block and to shape the obturator in such a way as to fill completely the slot in the block and to leave the interior of the block smooth and cylindrical when the obturator is in place.

The plaster cast is made in a small metal

box with mortised joints similar to the mold (Fig. 3). The box is .5" deep, .4" wide, and .75" long inside with 3/8" walls. A holder similar to (j) (Fig. 2) is used to support the cylinder (d) and the casting box. The guide (g) is not necessary in molding the obturators. When the box is filled with fine thoroughly mixed plaster the surface of the wax impression is painted with plaster to insure close adherence to the contour of the impression. The wax is then immersed, impression down, in the plaster. After setting, the excess plaster is chipped off and the wax removed after heating under hot tap water. Before filling the cast with lucite the surface of the plaster is painted with a sealing agent.<sup>||</sup> The filling and baking procedure is the same as described for the electrodes. The obturator is formed on the end of a lucite bar (f, Fig. 2, photo 3) and is sawed off after the plaster has been chipped off. (A weak solution of HCl is useful in removing the last bits of plaster). The obturator is then slipped into place on the electrode block. After

<sup>||</sup> L. D. Caulk Company, Milford, Dela. Al-Cote sealing agent.

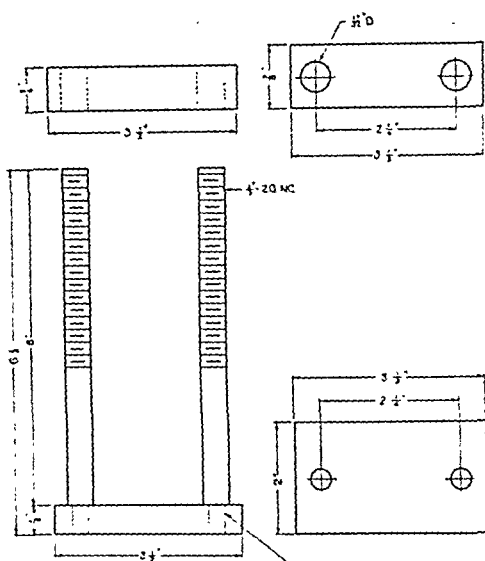


Fig. 7.

Detail drawing of press, (Fig. 2) (photo 2).

straight rubber tube until the hole in the side of the terminal coincides with the junction of the lumina. Pushing the terminal beyond this point and withdrawing to the proper position will produce a more reliable connection. Two or 3 turns of thread below the junction aids in securing the rubber to the terminal. Water-proof cement between the rubber tube and the lucite block insures good electrical insulation of the terminal. The hollow stainless steel terminal for the distal end of the tube is filled with mercury (Fig. 5). With a smooth 18-gauge needle and syringe 5 cc of mercury are flushed rapidly through the rubber tube and electrode terminal. Without allowing air to enter either tube and with the syringe in place the mercury in the distal terminal is joined by cohesion to the mercury in one lumen of the rubber tube and the terminal inserted. To insure complete filling the needle is re-

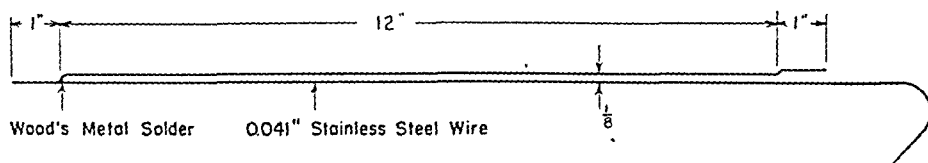


Fig. 8.

Diagram of stainless steel wires used for constructing double lumen latex leads.

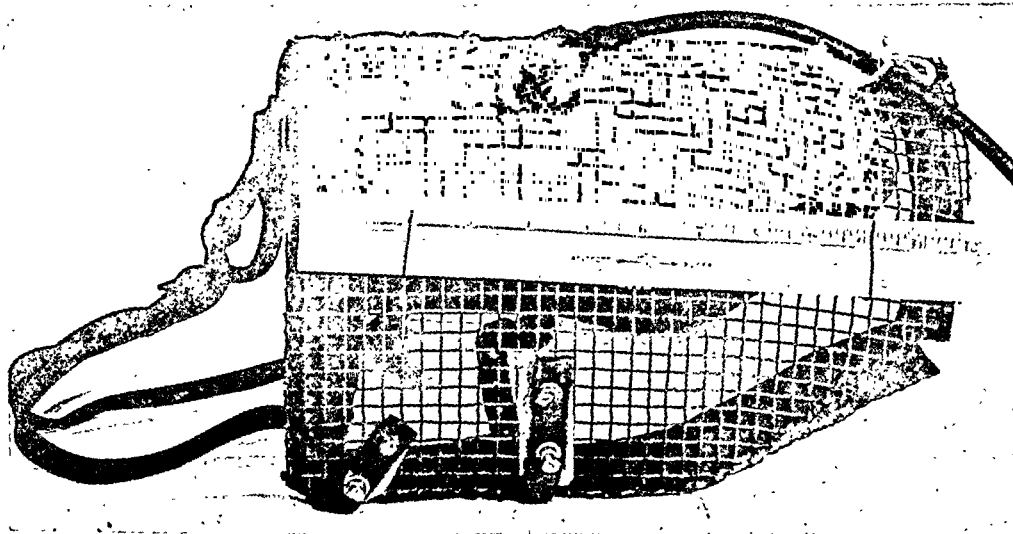


Fig. 9.

Photograph illustrating the gravel screen saddle, electrode terminal block, and lamp cord connecting the electrodes to the stimulator.

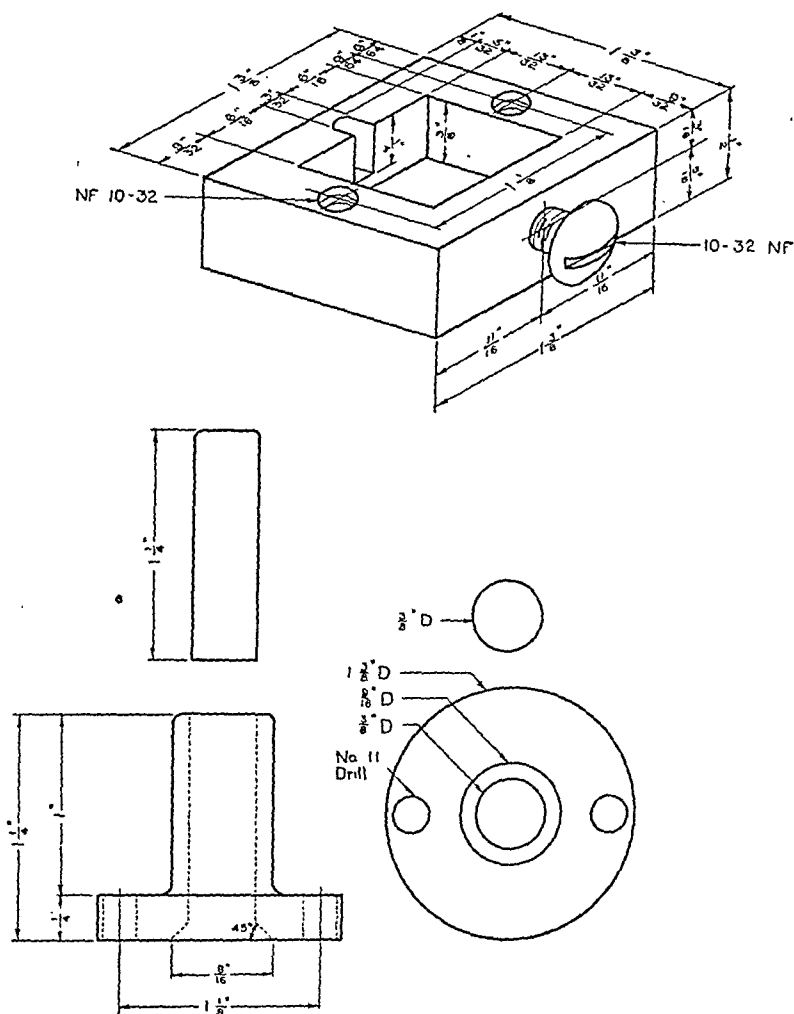


FIG. 6.  
Pictorial drawing of holder (j) and detail of cylinder (d) and piston (e) (Fig. 2).

tubes is controlled by the length of time allowed for deposition of latex on the wires. Usually 5 to 10 minutes is sufficient. The wires are carefully removed from the latex and approximately one hour allowed for coagulation to proceed. The wires and tubes are then washed in tap water for one hour, rinsed in distilled water, and dried until all white color has disappeared. Twenty-four hours at room temperature or 12 hours at 50°C will usually complete the drying. The tubes are finally cured for 20 minutes at 105°C. The tubes are removed quickly from the curing oven

and the wires separated at the Wood's metal junction. Later the wires are removed, the excess rubber is trimmed off, and the tubes cleaned with 1N HCl, water, alcohol, and dried with air. The tube extending past the junction of the lumina should be cut as short as possible to insure continuity of the junction of the tubes and the hole in the stainless steel terminal in the electrode block (Fig. 1).

*Step 5: Assembling the electrode and leads.* The hollow portion of the electrode terminal is filled with mercury with a syringe and needle. The terminal is inserted into the

tion could be made between urine samples containing different quantities of added digitoxin.

Ten albino rats (average weight 165 g) were given 100  $\mu$ g of digitoxin per kilogram of body weight by intravenous injection, and their urine was collected for 24 hours. The urine samples of 5 of these rats were also collected on the second and third day following injection. Twelve rats (average weight 223 g) were given 1000  $\mu$ g of digitoxin per kilogram of body weight and similar urine collections were made during the first 24 hours. Urine collections of eight of these rats also were made on the second and third days following injection. All urine samples were extracted and tested on the duck hearts as described above.

**Results.** The injection of a moderately large amount of digitoxin (*i.e.*, 100  $\mu$ g/kilo) into 10 rats was not followed by the detectable appearance of digitoxin in the 24-hour samples of urine of any of the rats either 24, 48, or 72 hours after the injection. In view of the fact that each rat actually received approximately 16.5  $\mu$ g of digitoxin, absence of as much as one microgram of digitoxin in the urine indicated that little or no digitoxin was excreted by the kidneys of these rats.

The urine samples, however, collected dur-

ing the first 24 hours in the 12 rats who had received 10 times the above amount of digitoxin (1000  $\mu$ g/kilo) contained an average of 6  $\mu$ g digitoxin (Range, 4 to 10  $\mu$ g). However, none of the urine samples collected during the second or third days after injection contained a detectable amount of digitoxin (*i.e.*, less than 1  $\mu$ g).

Thus, even in these rats which had received a relatively tremendous amount of digitoxin (average, 223  $\mu$ g) the average renal excretion of only 6  $\mu$ g (less than 3% of the administered dose) again indicates that the renal excretion of digitoxin in the rat is negligible. These results, however, are not to be construed as evidence for the negligible excretion of other cardiac glycosides in the rat. Moreover, these results may not be valid for other species in view of the fact that the rat appears to react anomalously to the administration of digitoxin.

**Conclusion.** A method for the quantitative determination of minute amounts of digitoxin in urine is described. The renal excretion of digitoxin is negligible in the rat.

The authors wish to express their appreciation to Vivian Seay and Fred Michaelis for their technical assistance.

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## 17206. Gonadotrophic Activity of Granules Isolated from Rat Pituitary Glands.\*

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The respiratory enzyme systems, cytochrome oxidase and succinoxidase, have been shown to be associated to a large extent with a large granule or mitochondrial fraction isolated by differential centrifugation of alkaline water homogenates (Schneider<sup>1</sup>), and saline extracts (Hogeboom, Claude and

Hotchkiss<sup>2</sup>) of rat liver. Subsequently morphologically intact mitochondria were isolated by differential centrifugation of 0.88 M sucrose homogenates of rat liver and kidney (Hogeboom, Schneider and Pallade<sup>3,4</sup>). On

<sup>2</sup> Hogeboom, G. H., Claude, A., and Hotchkiss, R. D., *J. Biol. Chem.*, 1946, **165**, 615.

<sup>3</sup> Hogeboom, G. H., Schneider, W. C., and Pallade, G. E., *Proc. Soc. Exp. Biol. and Med.*, 1947, **65**, 320.

<sup>4</sup> Hogeboom, G. H., Schneider, W. C., and Pallade, G. E., *J. Biol. Chem.*, 1948, **172**, 619.

\* Supported in part by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.

<sup>1</sup> Schneider, W. C., *J. Biol. Chem.*, 1946, **165**, 585.

moved with the rubber tube in the vertical position since the rubber will distend slightly due to the pressure of the mercury column. When the remaining rubber tube is completely filled with mercury a metal obturator is inserted and several turns of thread applied tightly around both the obturator and the distal terminal.

*Application of electrodes.* The splanchnic nerves are exposed through bilateral dorsal incisions. The nerves are carefully dissected free of surrounding tissue. An electrode is placed around each nerve and the obturator tied in place. Two or three turns of linen ligature around the rubber tube and through adjacent fascia holds the electrode in position so that the course of the nerve is altered as

little as possible. The mercury lead is then brought through the incision by a circuitous route and finally through the skin approximately two inches from the spine at about the level of the last rib. Anchoring the leads to bony structures and fascia at several points aids in securing the electrode in the proper position and prevents any pull directly on the nerve. Small flexible wire leads connect the mercury leads to a terminal block on the gravel screen saddle used to protect the leads (Fig. 9). The animal is tied to one end of a rectangular cage which allows him to lie down or stand at will but prevents turning completely around, thus preventing destruction of the leads to the stimulator.

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### 17205. Urinary Excretion of Digitoxin in the Rat.\*

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(With the Technical Assistance of Catherine S. Bland.)

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Heretofore it has not been possible to measure the actual renal excretion of digitalis glycosides. However, by means of the embryonic duck heart method, we have been able to measure in a quantitative fashion minute amounts of digitoxin (Sandoz) in the 24-hour urine volumes of the rat.

*Methods.* The 24-hour urine sample of a rat is concentrated to 10 ml volume by boiling. Three grams of infusorial earth are added and the mixture dried at 80° *in vacuo*. The dried residue is broken up with a glass rod, 50 ml of U.S.P. grade chloroform is added, and the mixture is shaken for one hour in a mechanical shaker. The chloroform extract is separated by filtration through paper. The residue on the paper is washed 3 times with fresh 10 ml portions of chloroform, the solvent being pressed out of the residue after each washing. The washings are combined

with the filtrate, the extract is concentrated to a small volume by boiling, and is then dried at 80°C *in vacuo*. The dried extract is taken up in 1 ml of ethanol by shaking for one hour. Ten ml of water is added to the alcohol and the solution is concentrated to 1 ml by boiling. Another 10 ml of water is added and the solution again concentrated to 1 ml. This concentrated solution is diluted to a final volume of 60 ml by the addition of 59 ml of Tyrode's solution (without glucose). Embryonic duck hearts are exposed to this latter solution and the time of "digitalis effect"<sup>1</sup> observed and the quantity of digitoxin estimated according to previously described methods.<sup>2</sup> It thus was found possible to detect as well as measure as little as one microgram of digitoxin added to the 24-hour urine sample of the rat. A quantitative differentia-

<sup>1</sup> Friedman, M., and Bine, R., Jr., *Proc. Soc. Exp. Biol. and Med.*, 1947, **64**, 162.

<sup>2</sup> Bine, R., Jr., and Friedman, M., *Am. J. Med. Sci.*, 1948, **216**, 534.

\* Aided by grants from The Life Insurance Medical Research Fund, The U. S. Public Health Service, and The Sandoz Chemical Works.

TABLE I.  
Gonadotrophic Activity of Fractions Obtained from Fresh Rat Pituitary Glands by High Speed Centrifugation.

Kind of fraction	Total amount of fraction injected, mg*	Normal rats (4 groups) Avg wt ovaries, mg	Castrate rats (1 group) Avg wt ovaries, mg
A. Whole homogenate	5 10 20	 126 (5) † 190 (9)	89 (3) † 153 (3)
B. Residue (nuclei and connective tissue)	20 100	15 (10) 24 (2)	18 (3) 47 (2)
C. Large granule fraction washed once with 0.88 M sucrose	10 20	 63 (7)	28 (3)
D. Large granule fraction washed twice with 0.9% NaCl	20	(A) 59 (3) † (B) 16 (6)	19 (3)
E. Washings from large granule fraction	20	31 (6)	33 (3)
F. Small granule fraction	10 20	 87 (6)	105 (3)
G. Small granule fraction washed twice with 0.9% NaCl	20	15 (3)	
H. Washings from small granule fraction	10 20	 50 (3)	100 (3) 214 (3)
I. Supernatant liquid from centrifuging at 15,000 × g	10 20	 44 (8)	97 (3)

\* Amount of fraction equivalent to the milligrams of fresh tissue indicated.

† Figure in parentheses indicates number of rats used in assays.

‡ The high value for the first two groups of rats (A) is probably due to incomplete extraction of the granules since a smaller volume of sodium chloride solution was used than for the granules of the other groups of rats (B).

ventional Warburg apparatus according to the method used in this laboratory (McShan and Meyer<sup>7</sup>).

**Results and discussion.** *Succinoxidase activity.* The large granule fractions C obtained from two groups of normal rats contained approximately 60% of the total succinoxidase activity found in the whole homogenates. Fraction C consisted largely of mitochondria as shown by examination of stained smears. In this connection it is of interest that Schneider,<sup>1</sup> and Hogeboom, Claude, and Hotchkiss<sup>2</sup> reported that 70 and 74%, respectively, of the succinoxidase activity of rat liver is present in the large granule fraction. Hogeboom, Schneider, and Pallade<sup>1</sup> prepared rat liver homogenates in 0.88 M sucrose and recovered 65 to 82% of

the succinoxidase activity in the mitochondria.

*Gonadotrophin.* The results obtained on assay of the various fractions of rat pituitary tissue are summarized in Table I. The results obtained with the whole homogenates designated as A in Table I show, as is already known, that rat pituitary glands contain a high level of gonadotrophin, and that the level of glands from castrate animals is greater than that of glands from normal rats. Very little gonadotrophic activity was retained in Fraction B, which consisted of various elements such as nuclei and connective tissue. These elements comprised a high percentage of the solid content of the glands.

The large granule fraction C consisted to a large extent of mitochondria as shown by the staining method of Bensley.<sup>6</sup> The small granule fraction F contained many fewer mitochondria than did fraction C. The re-

<sup>7</sup> McShan, W. H., and Meyer, R. K., *Arch. Biochem.*, 1946, 9, 165.



the basis of these results it seemed that it might be possible to isolate granule fractions from fresh pituitary tissue, and study them not only from the standpoint of their enzyme activity but also from the standpoint of whether gonadotrophic hormone is associated with them. Results of preliminary experiments using rat pituitary gland are given in this report.

*Experimental.* The pituitary glands were obtained from 4 groups of 50 to 60 normal adult rats (Holtzman strain) and one group of 35 adult rats castrated for 3 months. The animals were killed by decapitation. The glands were removed immediately, dissected free of the pars nervosa, and the adeno-hypophyses were placed on moist hard filter paper in a Stender dish which was kept on ice until the collection was completed. The glands were weighed and placed in a sharp pointed homogenizing tube that contained 0.4 ml of cold 0.88 M sucrose. The tube was kept in an ice bath at all times except during homogenization. The glands were finely homogenized in 19 volumes of 0.88 M sucrose which was the concentration of sucrose used by Hogeboom, Schneider and Pallade.<sup>3,4</sup>

The pituitary homogenates were fractionated by differential centrifugation in the multispeed head of an International refrigerated centrifuge kept at 2°C. The method of fractionation as to time and speed of centrifugation is similar to that reported by Schneider<sup>5</sup> for the isolation of mitochondria from isotonic sucrose (8.5 g sucrose per 100 ml) homogenates of rat liver. The liver mitochondria were washed with isotonic sucrose and they were shown to contain the major portion of the succinoxidase activity of the homogenates. It should be pointed out that the granule fractions isolated from the pituitary homogenates were washed with 0.9% NaCl and this resulted in the separation of the gonadotrophin from these fractions. The method of fractionation is as follows:

1. The whole homogenate designated as *A* was centrifuged at approximately 500 × g for 10 minutes. The opaque extract was removed and the residue was washed twice by suspending in 0.88 M sucrose and centrifuging in the same way. The washings were combined with

the above sucrose extract. The residue which consisted of cell nuclei and other insoluble material was designated as *B*.

2. The extract was centrifuged at 8,000 × g for 20 minutes. The aqueous sucrose extract was removed. The recovered precipitate (large granule fraction) was designated as *C*, and it was washed once by suspending in 0.88 M sucrose and centrifuging. The washings were added to the sucrose extract. The large granule fraction was suspended in 0.9% NaCl and centrifuged. This treatment was repeated one time. The washed fraction was suspended in NaCl and designated as *D*, and the combined NaCl washings as *E*.

3. Fractions containing small granules were recovered from the aqueous sucrose supernatant fractions of 2 groups of normal and the group of castrate rats by centrifuging for one hour at 15,000 × g. The small granule fraction was designated as *F*; it was washed twice with 0.9% NaCl. The washed granules were designated as *G*, and the washings as *H*. The aqueous sucrose extract from which the granules were removed was designated as *I*.

The fractions obtained by the above procedure were stained by a modification of the acid fuchsin methyl green method of Bensley<sup>6</sup> as an aid in estimating the homogeneity of the fractions.

The above fractions were assayed for gonadotrophic activity by use of 21-day-old female rats of the Holtzman strain. The volumes of the fractions were adjusted with saline for purposes of injection. Each rat received 0.5 ml of the proper fraction on the afternoon of the first day, and 0.5 ml on the morning and afternoon of the next 4 days. Autopsy was performed on the morning after the last injection. The ovaries were removed, dissected free of other tissues and weighed. The qualitative effect as to the presence of follicles and corpora lutea was recorded. The degree of stimulation of the uteri was also noted.

The succinoxidase activity of the whole sucrose homogenate and the large granule fraction was determined by the use of a con-

<sup>3</sup> Schneider, W. C., *J. Biol. Chem.*, 1948, **170**, 259.

<sup>6</sup> Bensley, R. R., *Am. J. Anat.*, 1911, **12**, 297.

TABLE I.  
Urinary Excretion of Amino-acid N and Glucose, and Balance N During Periods of Control and Insulin Insufficiency.\*

Period	Balance N, mg	Urine amino-acid N		Urine glucose, g
		mg	% of total urine N	
Normal control	+57.3	.961	.6	0
Diabetic + insulin	+14.4	1.24	.5	.869
Diabetic no insulin	-126.	1.29	.6	2.25

\* The values represent the average of 9-24 hr collections, 3 from each of 3 rats.

TABLE II.  
Amino-acids and Related Substances in Urines of Rats, Demonstrated by Paper Chromatography.

Amino-acids	No. of 24 hr urine samples containing the respective amino-acids		
	Normal control (9 samples)	Alloxan-diabetes + insulin (9 samples)	Alloxan-diabetes without insulin (9 samples)
Aspartic acid	6	5	2
Glutamic "	9	9	9
Glycine	9	9	8
Alanine	9	9	9
Valine	3	3	1
Leucine	3	2	1
Taurine	6	9	9
Glutamine	9	7	8
Unidentified polypeptide	5	5	5

excretion of amino-acids may vary considerably in conditions that involve disturbances of nitrogen metabolism in the body and alterations in renal function. It seemed of interest to determine whether ketonemic acidosis in the diabetic rat, with greatly increased cellular catabolism and losses of nitrogen from the body, would be characterized by significant changes in amino-aciduria.

*Methods.* Male Rockland Farm rats weighing approximately 250 grams were fed the diet mixture of Cox and Imboden.<sup>6</sup> The diet was thoroughly mixed to ensure uniformity of composition, especially with regard to the nitrogen content of aliquots. Adequate supplements of vitamins A and D were given at weekly intervals. The animals were placed in individual metabolism cages designed to permit measurement of food intake and for separate collection of the urine and feces. Urine specimens were collected under toluol and preserved by freezing. Alloxan (Bios)

was administered as a single dose, 50 mg per kg body weight, in 2.5% aqueous solution, injected intravenously after the rats were fasted 12 hours.

Total nitrogen determinations on diet, urine, and feces were performed by the micro-Kjeldahl method. Urea nitrogen was determined by the manometric method of Van Slyke and Kugel.<sup>7</sup> Amino-acid nitrogen was determined by the ninhydrin, carbon-dioxide method of Van Slyke, MacFadyen, and Hamilton.<sup>8</sup> The Nelson modification<sup>9</sup> of the Somogyi method was employed for the determination of glucose in blood and urine. Qualitative tests for acetone in the urine were made with a powdered mixture of sodium nitroprusside, sodium carbonate, and ammonium sulfate (Denco test). Paper chromatographic methods as described by Dent<sup>2,4</sup>

<sup>7</sup> Van Slyke, D. D., and Kugel, V. H., *J. Biol. Chem.*, 1933, **102**, 489.

<sup>8</sup> Van Slyke, D. D., MacFadyen, D. A., and Hamilton, P. B., *J. Biol. Chem.*, 1943, **150**, 251.

<sup>9</sup> Nelson, N., *J. Biol. Chem.*, 1944, **153**, 375.

<sup>6</sup> Cox, W. M., and Imboden, M., *Proc. Soc. Exp. Biol. and Med.*, 1936, **34**, 443.

sults given in Table I show that both these fractions contained gonadotrophin, which is similar to the results obtained by Catchpole<sup>8</sup> who reported that a major part of the gonadotrophin of sheep pituitary glands is found in the large granule fraction.

The large granule fraction C plus the small granule fraction F of glands from both normal and castrate animals contained more than 50% of the total gonadotrophin. The major part of the remaining hormone is found in the soluble fraction I, which may be accounted for either on the basis of the presence of small granules which were not removed by centrifugation, or by the solubility of the hormone in the sucrose. The greater part of the gonadotrophin was removed from the granule fractions C and F by extraction with 0.9% sodium chloride solution to give fractions E and H, respectively. The insoluble fractions (D and G) that remained after extraction contained little gonadotrophin. Whether the gonadotrophin was extracted from the gran-

ules or whether granules containing gonadotrophin were dissolved is not clear from present results. Further work is necessary to determine whether the hormone is associated with the mitochondria or with granules that are recovered with the mitochondria.

Conclusions cannot be made as to whether the FSH and LH activities are differentially distributed between the different fractions since all the active fractions produced follicles and corpora lutea.

*Summary.* Approximately 60% of the succinoxidase activity of rat pituitary glands were found to be associated with the large granule fraction, and more than 50% of the gonadotrophin of these glands was associated with two granule fractions isolated from sucrose homogenates by differential centrifugation. The supernatant fraction from which the granules were removed contained the remaining gonadotrophic activity. The gonadotrophin can be extracted from the isolated granules with isotonic sodium chloride solution.

<sup>8</sup> Catchpole, H. R., Abstract, *Fed. Proc.*, 1948, 7, 19.

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## 17207. Urinary Excretion of Amino-acids During Alloxan-induced Diabetes in Rats.\*

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The experiments reported here were undertaken to determine whether the metabolic disturbances of alloxan-induced diabetes in rats, with and without insulin-control, would entail an abnormal excretion of amino-acids in the urine.

Paper chromatographic methods developed by Consden and Martin<sup>1</sup> and adapted to urine analysis by Dent<sup>2-4</sup> have greatly facilitated

the qualitative detection of amino-acids. Several investigators applying these methods have gathered much new information on the abnormal amino-acidurias found in the Fanconi syndrome,<sup>2,3</sup> acute yellow atrophy of the liver,<sup>2</sup> and in progressive muscular dystrophy.<sup>5</sup> Results of studies on these unrelated disorders indicate that the patterns of urinary

\* Aided by a grant from the Nutrition Foundation, Inc.

<sup>†</sup> Postdoctorate Research Fellow, National Institute of Health, U. S. Public Health Service.

<sup>1</sup> Consden, R., Gordon, A. H., and Martin, A. J. P., *Biochem. J.*, 1944, 38, 224.

<sup>2</sup> Dent, C. E., Conference on Metabolic Aspects of Convalescence, Josiah Macy, Jr. Foundation, 1946, Nov. 12-13, 126.

<sup>3</sup> Dent, C. E., *Biochem. J.*, 1947, 41, 240.

<sup>4</sup> Dent, C. E., *Biochem. J.*, 1948, 43, 169.

<sup>5</sup> Ames, S. R., and Risley, H. A., *Proc. Soc. EXP. BIOL. AND MED.*, 1948, 68, 131.

Seibert *et al.*<sup>4,5</sup> have found that the carbohydrate content of human serum proteins increases in cancer and tuberculosis, and Niazi and State<sup>6</sup> have confirmed this elevation in cancer and infectious diseases. These findings are in accord with similar results reported in the earlier literature by other investigators. Meanwhile, it was shown by Glick and co-workers that a similar elevation in the level of hyaluronidase inhibitor (not the inhibitor that is an antibody to hyaluronidase) occurs in cancer,<sup>7</sup> and in a wide variety of both virus and bacterial infections.<sup>8-11</sup> Increases of hyaluronidase inhibitor in infectious diseases were also reported by Thompson,<sup>12</sup> Friou and Wenner,<sup>13</sup> and others.

It could reasonably be supposed that the hyaluronidase inhibitor might be associated with mucoprotein or carbohydrate compounds that could compete with hyaluronic acid for the enzyme. This possibility appeared to be strengthened by the electrophoretic studies of Winzler *et al.*,<sup>1</sup> which indicated that the isolated mucoprotein migrated nearer to the albumin fraction than to the other plasma proteins at pH 8.3, and of Glick and Moore,<sup>14</sup> which showed that the hyaluronidase inhibitor in the serum migrated chiefly with the albumin at pH 8.6.

These considerations, along with the ob-

served parallelism in the elevation of both the inhibitor and the mucoprotein in the same diseases, prompted the present investigation of the possibility of their association or identity.

Mucoprotein samples of human serum prepared from perchloric acid filtrates by the procedure of Winzler *et al.*<sup>1</sup> showed little inhibitory activity per gram of protein compared to serum. Plasma mucoprotein preparations isolated by an ammonium sulfate procedure<sup>15</sup> which were electrophoretically homogeneous also had very little hyaluronidase inhibitor activity. The very low isoelectric points of plasma mucoproteins permit their electrophoretic separation from other plasma proteins at pH 4 or 4.5.<sup>16,17</sup> We have isolated plasma mucoproteins electrophoretically and tested them for hyaluronidase inhibitor. One such sample which was isolated, dialyzed, and lyophilized had somewhat more inhibitory effect but was still much weaker than fresh serum on an equal total protein basis. Adding magnesium ions to these preparations had no influence.

In view of the instability of the hyaluronidase inhibitor, it was not felt that its lack of identity with plasma mucoproteins was established by the foregoing results. The experiment shown in Table I, however, shows more conclusively that hyaluronidase inhibitor is not associated with the acidic plasma mucoproteins.

A pooled sample of serum freshly obtained from several patients with lobar pneumonia was adjusted to pH 4.5 with approximately 2 N acetic acid. A sample was removed for the hyaluronidase inhibitor determination, and the remainder of the serum was centrifuged to remove the small amount of precipitate which had formed. This clarified serum was then immediately subjected to electrophoresis, without any preliminary dialysis, against an acetate buffer in sodium chloride. The total acetate concentration was 0.02 M,

<sup>4</sup> Seibert, F. B., Seibert, M. V., Atno, A. J., and Campbell, H. W., *J. Clin. Invest.*, 1947, **26**, 90.

<sup>5</sup> Seibert, F. B., Pfaff, M. L., and Seibert, M. V., *Arch. Biochem.*, 1948, **18**, 279.

<sup>6</sup> Niazi, S. A., and State, D., *Cancer Res.*, in press.

<sup>7</sup> Hakanson, E. Y., and Glick, D., *J. Nat. Cancer Inst.*, 1948, **9**, 129.

<sup>8</sup> Glick, D., and Gollan, F., *J. Inf. Dis.*, 1948, **83**, 200.

<sup>9</sup> Grais, M. L., and Glick, D., *J. Invest. Dermatol.*, 1948, **11**, 259.

<sup>10</sup> Glick, D., and Campbell, B., *Proc. Soc. Exp. Biol. and Med.*, 1949, **70**, 29.

<sup>11</sup> Grais, M. L., and Glick, D., *J. Inf. Dis.*, in press.

<sup>12</sup> Thompson, R. T., *J. Lab. Clin. Med.*, 1948, **33**, 919.

<sup>13</sup> Friou, G. J., and Wenner, H. A., *J. Inf. Dis.*, 1947, **80**, 185.

<sup>14</sup> Glick, D., and Moore, D. H., *Arch. Biochem.*, 1948, **19**, 173.

<sup>15</sup> Weimer, H., Mehl, J. W., and Winzler, R. J., submitted for publication.

<sup>16</sup> Petermann, M. P., and Hogness, K. R., *Cancer*, 1948, **1**, 104.

<sup>17</sup> Mehl, J. W., Golden, F., and Winzler, R. J., submitted for publication.

were used to detect and identify individual amino-acids in all urines.

*Experimental.* The results summarized here were obtained on 3 rats, each studied in 3 metabolic periods of 3 or more days: I, in normal state during preliminary control periods; II, in the diabetic state following the injection of alloxan, treated with adequate doses of insulin, and III, after withholding insulin. Severely diabetic states were induced in every animal, as indicated by the rapidity of the development of acetoneuria, acidosis and death within 3 to 5 days when insulin was withheld. The preliminary period I included three consecutive 24-hour collections of urine. After the administration of alloxan the ensuing diabetic state was controlled by daily injections of 1 to 3 units of protamine zinc insulin, the doses being adjusted to maintain the body weight constant and to keep the urine acetone-free. After the establishment of satisfactory control, 24-hour collections of urine were resumed and continued through periods II and III, until the animal died.

Data from 3 rats are presented in Table I. The individual amino-acids identified by the chromatographic method in the urines of the 3 animals in respective periods are listed collectively in Table II.

*Discussion.* During periods of severe and

fatal acidosis in the alloxan-diabetic rat, deprived of insulin, the pattern of excretion of amino-acids in the urine remained essentially unchanged, compared with periods of control. The ratio of amino-acid nitrogen to total nitrogen excreted in the urines remained relatively constant, independent of the development of ketosis, acidosis, and increased excretion of total nitrogen. These facts suggest the conclusion that although protein catabolism is increased in alloxan-induced diabetes during periods of insulin insufficiency, normal metabolic pathways are utilized, and amino-acids are efficiently conserved by the kidney. This is in accord with the results of studies on human diabetics as reported by Hall.<sup>10</sup>

*Summary.* Nitrogen balance studies on 3 male rats during periods of normal control, of alloxan-induced diabetes with insulin therapy, and of fatal acidosis after withholding insulin, are reported.

The pattern of excretion of urinary amino-acids, and the ratio of total amino-acid nitrogen to total urinary nitrogen remained essentially unchanged throughout the 3 periods, including the terminal stage with greatly increased losses of total nitrogen.

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<sup>10</sup> Hall, D. A., *Biochem. J. Proceedings*, 1948, 43, lvii.

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## 17208. Lack of Identity of Hyaluronidase Inhibitor and Certain Mucoproteins in Blood Serum.\*

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Winzler and co-workers<sup>1,2</sup> observed that

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\* Aided by a grant from the National Cancer Institute, U. S. Public Health Service, Bethesda, Md., and by the Committee on Growth of the National Research Council, acting for the American Cancer Society.

<sup>1</sup> Winzler, R. J., Devor, A. W., Mehl, J. W., and Smyth, I. M., *J. Clin. Invest.*, 1948, 27, 609.

elevations of plasma mucoprotein levels occur in patients with cancer and pneumonia. Prinzmetal *et al.*<sup>3</sup> have noted a similar increase in patients with myocardial infarctions.

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<sup>2</sup> Winzler, R. J., and Smyth, I. M., *J. Clin. Invest.*, 1948, 27, 617.

<sup>3</sup> Simpkin, B., Bergman, H. C., and Prinzmetal, M., *Am. J. Med.*, 1949, in press.

TABLE II.  
Mucoprotein and Hyaluronidase Inhibitor Levels in "Lipoid Nephrosis."

Group of children	No.	Age range	Mean mucoprotein-tyrosine $\pm$ std. error of mean (mg %)	Mean % hyaluronidase inhibition $\pm$ std. error of mean per 0.02 ml fresh serum
Normal	40	1-15		21.2 $\pm$ 0.9
"	55	1-15	2.2 $\pm$ 0.1	
Nephrosis	9		1.5 $\pm$ 0.1	56.6 $\pm$ 3.7

TABLE III.  
Hyaluronidase Inhibitor and Mucoprotein Levels in Miscellaneous Conditions.

Disease of child	Age	Mucoprotein-tyrosine (mg %)	% hyaluronidase inhibition per 0.02 ml fresh serum
Acute poliomyelitis	12	4.2	44
Acute lymphatic leukemia	8	9.4	29
Metastatic neuroblastoma	1.3	9.8	56
Inactive rheumatic fever	16	5.1	11
Upper respiratory infection	11	2.6	31
Normal	5	3.8	23
"	12	2.6	33

children with nephrosis, which will be reported in detail elsewhere by Good, Kelley, and Glick, a further lack of correlation between the mucoprotein and inhibitor content of untreated sera was found (Table II). In this series the mucoprotein values measured by the method of Winzler *et al.*<sup>1</sup> were 32% less than the normal, while the inhibitor values were 167% greater than the normal. In other diseases, too, widely divergent serum values were found, as illustrated by a few examples given in Table III.

It is therefore clear that there is no identity of the hyaluronidase inhibitor and the acidic mucoproteins of blood serum as separated by electrophoresis at pH 4.5, by precipitation from perchloric acid filtrates, or by ammonium sulfate fractionation. This does not negate the possibility that the inhibitor may be found in other mucoprotein or glycoprotein components, nor is it at variance with the striking statistical correlation that has been observed between the elevation of serum mucoprotein and hyaluronidase inhibitor in cancer and infectious diseases.

*Summary and conclusions.* 1. Electrophoretically and chemically separated fractions of human serum containing high concentrations of mucoproteins showed no increase in non-specific hyaluronidase inhibitor when compared to native serum or other mucoprotein-poor fractions of serum.

2. In children suffering from "lipoid nephrosis" the hyaluronidase inhibitor levels of the serum were higher while the mucoprotein levels were significantly lower than normal.

3. In spite of the striking statistical correlation existing between the serum levels of mucoprotein and non-specific hyaluronidase inhibitor in a wide variety of human diseases, marked disparity in the serum levels of these substances occasionally occurs.

4. On the basis of these findings the lack of identity of the non-specific hyaluronidase inhibitor and these serum mucoproteins is pointed out.

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TABLE I.  
 Hyaluronidase Inhibitor in Mucoprotein Concentrates.

Sample	mg % mucoprotein-tyrosine	% hyaluronidase inhibition per 0.02 ml
Normal serum	2.2	21.2
Pooled pneumonia serum frozen immediately	14.0	59
Same adjusted to pH 4.5 and frozen	12.0	11
Same adjusted to pH 4.5 centrifuged and supernatant frozen	11.8	8
Same adjusted to pH 4.5 centrifuged and supernatant held in electro- phoresis bath during experiment	13.3	5
Compartment I (acidic mucoproteins)	11.4	8
Compartment II (normal globulin and mucoprotein with reduced globulin)	10.5	13
Compartment III (globulins, no mucoprotein)	0.8	8
Compartment IV. Normal albumins and globulins (reduced mucoprotein)	6.7	6

the ionic strength 0.1, and the pH was 4.5 at 22°C. A 4-compartment cell was employed, and compensation was used as needed to keep the upper edge of the albumin boundary at the middle junction of the cell on the side towards the anode. The electrophoresis experiment was terminated when the fastest mucoprotein component had reached a point about 2/3 of the distance up the upper half of the anodic limb (compartment I). At the same time, the most rapidly moving globulin had reached the top of the upper half of the cathodic limb (compartment III).

At least 3 positively-charged components migrated toward the negative electrode—the fastest with a mobility corresponding to the major mucoprotein component. Compartment I contained only these acidic components and when removed, the concentration of these components should have been 1/2 to 2/3 that in the serum. Compartment II was the lower half of this arm and contained mucoprotein and albumin in the original concentration with somewhat reduced amounts of globulin. Compartment III was the upper section of the arm ascending to the negative electrode and contained the globulins and very little albumin or mucoprotein. Compartment IV

was the lower section of this arm and contained globulins and albumin at original concentration with a reduced mucoprotein concentration. These samples were frozen immediately after the electrophoretic separation and were kept frozen until analysis of hyaluronidase inhibitor in order to prevent deterioration. Appropriate controls were also carried as is indicated in Table I. The mucoprotein levels were carried out using the tyrosine and carbohydrate determinations previously described<sup>1</sup> and the hyaluronidase inhibitor was determined as described earlier.<sup>8,18</sup> The data of Table I show that there is no correlation between the hyaluronidase inhibitor activity and the mucoprotein content of the various samples—the sample from compartment III, containing little or no acidic mucoprotein, having as much inhibitor as other compartments. The partial inactivation of the inhibitor resulting from adjustment to pH 4.5 does not seriously affect our conclusion that the hyaluronidase inhibitor and acidic plasma mucoproteins are not associated.

During the course of a study on serum from

<sup>18</sup> Wattenberg, L., and Gliek, D., *J. Biol. Chem.*, in press.

possible from complete polymerization of the dextrose component.

The inocula were prepared by incubating the cultures for 24 hours at room temperature in 5 ml of the complete basal medium (Table I) to which biotin was added in the low concentration of 0.01  $\mu$ g per liter. The bacterial cells were centrifuged from the medium, resuspended in a double volume of sterile saline, recentrifuged and again resuspended in sterile saline to make a 1:12 dilution of the original culture. By means of a sterile syringe (20-gauge needle) one drop of this diluted inoculum was added to each 3 ml tube of medium. In the case of experiments involving *Leuconostoc* the tubes after inoculation were incubated for 72 hours at a constant temperature of 25° C.

As an organism requiring biotin *Lactobacillus arabinosus* 17-5 was also employed in this investigation for the purpose of comparison and assay. The culture was grown in the same basal medium and the inoculum was prepared in the same manner as described for the *Leuconostoc*. Each tube, however, was incubated at 37° C for 72 hours.

The rate of growth of all organisms was measured by titration of each tube of medium with standard alkali, bromthymol blue being used as indicator. The results, corrected for the blank titration of uninoculated controls, are expressed in terms of ml of 0.01 N acid produced per ml of culture.

**Results and Discussion.** The presence of biotin in sucrose has been reported by various investigators.<sup>3,4</sup> To verify the observation that *Leuconostoc* require biotin for growth in glucose and fructose media, but not in a sucrose medium, a sample of the disaccharide was hydrolyzed with acid to invert sugar. Kreuger and Peterson<sup>5</sup> have shown that autoclaving for 2 hours at 15 lbs pressure with 2 N acid did not destroy biotin added to media. It therefore seemed safe to assume that the conditions employed here, 80 minutes

refluxing at atmospheric pressure with 0.1 N acid, would not destroy any biotin present in the sucrose samples.

The ability of the 3 strains of *Leuconostoc* to grow in media containing sucrose, or the same sucrose hydrolyzed by acid, was determined both in the presence and absence of added egg white. The results given in Table II show that whereas satisfactory growth occurred both in the sucrose and hydrolyzed sucrose media, added egg white was strongly inhibitory in the medium containing invert sugar. In contrast, in the sucrose medium, egg white inhibited growth only partially and then to a degree approximately inversely proportional to the yield of dextran. *L. dextranicum* 8086 with a dextran yield of 33%, was markedly inhibited by egg white, whereas *L. dextranicum* elai, which forms a theoretical yield of the polysaccharide, was insensitive to the biotin-binding action of egg white. *L. mesenteroides* 683 occupied a middle position both with respect to dextran yield and inhibition by egg white. Thus, to the degree that a strain of *Leuconostoc* utilizes sucrose via the mechanism resulting in dextran synthesis, it appears to be free of a requirement for biotin.

The ability of the *Leuconostoc* strains to grow in glucose or fructose media in the presence or absence of egg white also was determined. The low level of growth observed in the basal media without added biotin was suppressed by egg white in the cases of *L. dextranicum* 8086 and *L. mesenteroides* 683. Growth of *L. dextranicum* elai was increased by the added egg white. Shorb<sup>6</sup> has reported the presence of small amounts of vitamin B<sub>12</sub> and the T. J. factor in egg white. In separate experiments, *L. dextranicum* elai was found to be stimulated slightly by vitamin B<sub>12</sub><sup>†</sup> and to respond strongly to tomato serum as a source of the T. J. factor. The observed stimulation by egg white thus appears to be due to its T. J. factor content.

Reversal of egg white inhibition by added biotin also was demonstrated. In order to

<sup>3</sup> Jackson, W. R., and Macek, T. J., *Ind. Eng. Chem.*, 1944, **36**, 261.

<sup>4</sup> Hall, H. H., Paine, H. S., and Fabian, F. W., *Food Research*, 1947, **12**, 99.

<sup>5</sup> Kreuger, K. K., and Peterson, W. H., *J. Bact.*, 1948, **55**, 693.

<sup>6</sup> Shorb, M. S., *Science*, 1948, **107**, 397.

<sup>†</sup> We are indebted to Merek and Company, Rahway, N. J., for a sample of crystalline vitamin B<sub>12</sub>.



# 17209. Biotin-Carbohydrate Interrelationships in the Metabolism of *Leuconostoc*.\*

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(Introduced by Emmett B. Carmichael.)

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During an investigation of the direct utilization of sucrose by *Leuconostoc*<sup>1</sup> it was found that the organisms apparently did not require biotin when the disaccharide was employed, but the presence of the vitamin was essential for growth when the constituent monosaccharides were used in the media. Because a variation in vitamin requirement as a function of carbohydrate source was not found reported in the literature, and because it appeared to offer the possibility of recognition of a new function of biotin, the observation was investigated further.

**Experimental.** The basal medium employed had the composition given in Table I. With the exception of the carbohydrate component, the basal medium was made up in 3/2 strength, distributed in 2 ml amounts in 10 × 100 mm test tubes closed with cotton plugs, and sterilized 5 minutes at 15 lb. pressure. The carbohydrate component was made up in triple strength, sterilized by Seitz filtration, and added, aseptically, in 1 ml volumes. If other additives were involved, *e.g.*, oleic acid or egg white, these were included in the carbohydrate solution so that the final volume per tube remained constant at 3 ml.

Four sugar sources were employed: Sucrose, glucose, fructose and acid hydrolyzed sucrose. In the case of the first 3 sugars, the final concentration of 5% consisted of 90% of Seitz sterilized sugar and 10% of the same sugar sterilized in neutral phosphate buffer for 15 minutes at 15 lb. pressure. The resulting 0.5% concentration of heat sterilized sugar served as a source of the stimulatory materials reported to be necessary for the growth of many lactic acid organisms.<sup>2</sup> In

TABLE I.  
Composition of Basal Medium.

Constituent	Conc. per liter	
	g	mg
Carbohydrate	50	
Casein hydrolysate	5	
Cysteine		100
Tryptophane		100
Phenylalanine		50
Tyrosine		50
Adenine		10
Xanthine		10
Guanine		10
Thymine		10
Uracil		10
Nicotinic acid		1
Riboflavin		0.5
Thiamin chloride		0.5
Calcium pantothenate		0.5
Pyridoxine		0.4
p-Aminobenzoic acid		0.1
Folic acid		0.01
NaOAc	25	
NH <sub>4</sub> Cl	5	
MgSO <sub>4</sub> · 7H <sub>2</sub> O		100
NaCl		10
FeSO <sub>4</sub>		10
MnSO <sub>4</sub>		10
KH <sub>2</sub> PO <sub>4</sub>		500
K <sub>2</sub> HPO <sub>4</sub>		500

the remaining instance sucrose was hydrolyzed with 0.1 N HCl under reflux for 80 minutes, neutralized with KOH and passed through a Seitz filter.

Three strains of *Leuconostoc* were used in this investigation: *L. mesenteroides* 683, *L. dextranicum clai*, and *L. dextranicum* 8086 (ATCC). Of the various strains available these were selected because of the wide variation in their respective dextran synthesizing capabilities. *L. dextranicum clai* consistently gives yields of dextran approximating complete conversion of the dextrose half of the sucrose molecule into the polysaccharide. The yields routinely obtained with *L. mesenteroides* 683 and *L. dextranicum* 8086 were, respectively, 65% and 33% of that theoretically

\* Presented in part before the thirty-third annual meeting, Federation of American Societies for Experimental Biology, Detroit, April 1949.

<sup>1</sup> Carlson, W. W., and Whiteside-Carlson, V., *Fed. Proc.*, 1949, **8**, 189.

<sup>2</sup> Orla Jensen, S., *J. Soc. Chem. Ind.*, 1933, **52**, 374.

TABLE III.  
Assay of Biotin Level of Various Carbohydrate Media with *Lactobacillus arabinosus*.

Additions to basal medium	ml 0.01 N NaOH per ml medium			
	Sucrose	Hydrolyzed sucrose	Glucose	Fructose
None	1.4	4.3	1.7	0.7
Biotin 0.00005 $\mu$ g per ml	4.6	8.8	4.0	5.2
Biotin 0.0005 $\mu$ g per ml	19.0	23.3	23.3	16.6
Egg white 0.2 ml per tube	0.0	0.2	0.2	0.1
Egg white 0.2 ml per tube Biotin 0.01 $\mu$ g per ml	23.3	25.2	26.7	20.1

way leading to polysaccharide synthesis.

An attempt was made to estimate the amount of the vitamin in the various carbohydrate media by means of a standard biotin assay organism, *Lactobacillus arabinosus* 17-5. The results, briefly summarized in Table III, show that only a low level of growth was obtained with any of the carbohydrate media. Egg white suppressed this low level of growth, complete reversal of the inhibition being observed with added vitamin. Added to the basal media at a level of 0.00005  $\mu$ g per ml, biotin caused a definite stimulation of growth, a nearly maximal effect being obtained at a level of 0.0005  $\mu$ g per ml. These results indicate that *Leuconostoc* respond to very low levels of biotin.

To test the possibility that *Leuconostoc* synthesize biotin, the cultures were grown in the sucrose and invert sugar basal media for 72 hours. The cultures were then sterilized for 15 minutes at 15 lb pressure and aliquots of the resulting solutions added to tubes of the glucose basal medium, after which they were inoculated with *L. arabinosus*. Representative titration figures, as obtained with the material from the growth of *L. mesenteroides* 683 in sucrose and invert sugar media were, respectively, 1.5 ml and 5.2 ml. Thompson<sup>9</sup> has reported that organisms that syn-

thesize biotin release the vitamin into the medium. Since essentially no stimulation of the growth of *L. arabinosus* by *L. mesenteroides* culture solutions was observed, it appears unlikely that *Leuconostoc* synthesize biotin. It is therefore concluded that the observed difference in the biotin requirement of *Leuconostoc* in a sucrose medium, as compared with the requirement in media containing the constituent monosaccharides, is real. Information concerning the metabolic pathway in invert sugar, glucose, or fructose media, as compared with the pathway in a sucrose medium which leads to polysaccharide synthesis, thus may suggest a function for biotin in addition to those presently known.<sup>10</sup>

**Summary.** With 3 strains of *Leuconostoc* it was found that biotin was required for growth in media containing invert sugar, glucose, or fructose. In a sucrose medium the organisms were free of a biotin requirement to the degree that the disaccharide was utilized via the mechanism resulting in dextran synthesis.

<sup>9</sup> Thompson, R. C., Univ. of Texas Publ. 4237, 1942, p. 87.

<sup>10</sup> Couch, J. R., Cravens, W. W., Elvehjem, C. A., and Halpin, J. G., *Arch. Biochem.*, 1949, **21**, 77.

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TABLE II.  
Effect of Egg White, Biotin and Oleic Acid on the Growth of *Leuconostoc* in Various Carbohydrate Media.

Additions to basal medium	Test organism	ml 0.01 N NaOH per ml medium			
		Sucrose	Hydrolyzed sucrose	Glucose	Fructose
None	<i>L. dextranicum</i> 8086	12.3	9.7	1.1	1.5
	<i>L. mesenteroides</i> 683	9.7	11.6	1.5	2.0
	<i>L. dextranicum</i> elai	7.2	5.8	0.7	0.3
Egg white 0.2 ml per tube	<i>L. dextranicum</i> 8086	2.4	0.7	0.1	0.1
	<i>L. mesenteroides</i> 683	5.7	0.4	0.6	0.4
	<i>L. dextranicum</i> elai	7.1	2.5	1.9	2.6
Egg white 0.2 ml per tube	<i>L. dextranicum</i> 8086	—	12.0	11.2	8.5
	<i>L. mesenteroides</i> 683	—	7.2	7.7	13.4
	<i>L. dextranicum</i> elai	—	7.3	3.5	7.8
Biotin 0.01 μg per ml					
Oleic acid 5 μg per ml	<i>L. dextranicum</i> 8086	11.6	8.4	4.0	2.5
	<i>L. mesenteroides</i> 683	11.9	11.3	4.7	6.2
	<i>L. dextranicum</i> elai	0.2	0.9	0.1	0.9
Egg white 0.2 ml per tube	<i>L. dextranicum</i> 8086	8.6	0.2	0.1	0.1
	<i>L. mesenteroides</i> 683	7.7	0.3	0.1	0.2
	<i>L. dextranicum</i> elai	1.9	2.0	0.2	0.9
Oleic acid 5 μg per ml					

eliminate variables such as activity of the inoculum, the data for Table II were all collected at one time. Tubes corresponding to the addition of both egg white and biotin to the sucrose medium were not included in this experiment. From results of other experiments, however, it can be stated that added biotin reverses such variable inhibition of growth in sucrose media as occurs with the various strains of *Leuconostoc*. Slight stimulation over the growth observed in the basal medium without added biotin was customarily found, traceable to the presence of stimulatory factors in egg white.

Oleic acid is regarded as a probable product of biotin activity and is stated to be capable of substituting for the vitamin in the case of certain lactic acid organisms.<sup>7,8</sup> The effect of oleic acid, at a level of 5 μg per ml, was determined for the various types of carbohydrate media given in Table II. *L. dextranicum* elai showed a marked sensitivity to

oleic acid, growth being almost completely inhibited in all the media employed. With the other two strains of *Leuconostoc*, oleic acid produced little effect in sucrose or hydrolyzed sucrose media. Growth in glucose and fructose was stimulated, demonstrating an ability of the substance to partially replace the biotin requirement of the organisms. Reversal of egg white inhibition by added oleic acid occurred only with *L. mesenteroides* 683 and *L. dextranicum* 8086 in sucrose medium. In the case of *L. dextranicum* elai, the toxicity of oleic acid was reversed to a small extent by added egg white. Failure of oleic acid to reverse egg white inhibition in media containing invert sugar, glucose, or fructose, as compared with the result obtained in the sucrose medium, suggests that utilization of the intact disaccharide follows a different metabolic pathway.

The results given in Table II are taken to indicate that sucrose does contain traces of biotin, these small amounts of the vitamin being important to growth of the organisms in the invert sugar medium, and also in the sucrose medium to the extent that utilization of the disaccharide does not follow the path-

<sup>7</sup> Williams, V. R., and Fieger, E. A., *J. Biol. Chem.*, 1946, **166**, 335.

<sup>8</sup> Boquist, H. P., and Snell, E. E., *J. Biol. Chem.*, 1948, **173**, 435.

TABLE I.  
Changes of Blood Pressure and Electrocardiogram Due to Dihydroergocornine.

No.	Age	Sex	Diagnosis	Blood pressure			Electrocardiogram	
				Initial	5 min.	30 min.	Before	After
1*	57	F	Lactic acidosis	150/40	126/32	134/40	Left ventricular hypertrophy	No changes
2	60	F	Hypertensive cardiovascular Congestive heart failure	174/110	154/94	156/99	" "	" "
3*	64	F	Hypertensive cardiovascular Cerebral thrombosis	244/126	222/116	230/122	" "	" "
4	66	M	Generalized arteriosclerosis Diabetes mellitus Aortitis	190/86	172/78	156/82	" "	" "
5	47	F	Hypertensive cardiovascular Cerebral thrombosis	248/134	234/128	210/120	Left ventricular hypertrophy	" "
6	55	M	Hypertensive cardiovascular	168/138	146/114	150/116	" "	" "
7	62	M	Hypertensive cardiovascular Diabetes mellitus Pulmonary emphysema	172/96	144/88	138/88	" "	" "
8	42	M	Essential hypertension in malignant phase	184/136	160/126	162/120	" "	" "
9	32	M	Malignant hypertension	178/126	146/108	150/100	" "	" "
10	56	M	Hypertensive cardiovascular Lactic acidosis Congestive heart failure	224/132	186/112	184/112	Left ventricular hypertrophy PR 0.22	" "
11	65	M	Hypertensive cardiovascular Carcinoma of urinary bladder	198/108	178/99	166/96	Left ventricular hypertrophy (early)	" "
12	67	F	Hypertensive cardiovascular Arteriosclerosis	154/116	148/98	148/98	Left ventricular hypertrophy Multifocal extrasystoles	" "
13	47	F	Lactic acidosis Congestive heart failure	252/104	204/90	198/92	Left ventricular hypertrophy PR 0.23	" "

## 17210. Effect of Dihydroergocornine on the Heart.\*

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In a previous study it was shown that 0.5 mg of ergotamine tartrate or dihydroergotamine 45 injected intravenously would cause inverted T waves in patients with organic heart disease to revert to normal.<sup>1</sup> Seven of the 19 patients studied developed these significant electrocardiographic changes under the influence of these drugs and in 5 patients severe anginal pain occurred. This effect was attributed to a direct vasoconstrictor action of the ergotamine preparations on the coronary arteries. The experiments were repeated after blockade of the autonomic ganglia with tetraethylammonium chloride. Six of the 10 patients who received ergotamine tartrate in addition to tetraethylammonium chloride developed normalization of the previously inverted T waves. Changes in the repolarization of the heart muscle induced by the coronary vasoconstriction were assumed to be responsible for these electrocardiographic changes.<sup>2</sup>

The present study was undertaken to show the influence of dihydroergocornine (DHO 180) on the electrocardiogram of patients with left ventricular hypertrophy secondary to hypertension or aortitis.

**Material and Method.** Of the 24 patients examined 6 had a history of anginal pain and 5 had syphilitic aortitis with marked electrocardiographic changes so that involvement of the coronary ostia could be assumed to exist. All patients had the pattern of left ventricular hypertrophy with a depressed RS-T segment and inverted T waves in lead I and high R waves with similar changes of RS-T and T in lead CF<sub>5</sub>.

After 30 minutes of bed-rest a control electrocardiogram and the blood pressure were

recorded. Then 0.5 mg of dihydroergocornine was injected intravenously and the blood pressure readings and the electrocardiogram were repeated after 1, 5, 15, and 30 minutes. Standard leads as well as CF<sub>2</sub> and CF<sub>3</sub> were taken.

**Results.** In all cases the electrocardiogram remained unchanged throughout the period of investigation. The position of the RS-T segment did not change and the inversion of the T waves remained the same (Table I). The blood pressure fell in most patients and this fall seemed to be more pronounced the higher the initial blood pressure was.

The constancy of the electrocardiographic pattern was even more remarkable since 11 of the 24 patients were 65 years old or older. Anginal pain did not appear even in those patients who had suffered from it before admission. No untoward symptom or reaction developed in any of the 24 patients. Careful observation did not reveal any changes apart from the change in the blood pressure.

**Discussion.** This investigation was undertaken because of the report that dihydroergocornine does not produce vasoconstriction in the sympathectomized limb and represents the first pure sympatholytic derivative of ergot.<sup>3</sup> It belongs to the group of ergot alkaloids which combine a peripheral sympatholytic action on the receptors in the vessels with a central nervous effect which diminishes vascular tone.<sup>4</sup>

In view of the recommendation of this drug for the treatment of peripheral vascular diseases and certain forms of hypertension<sup>4</sup> it seemed desirable to ascertain whether it promotes anginal pain and whether it has a constrictor effect on the coronary arteries. Our studies indicate that such effects are absent and that in contrast to ergotamine tartrate and dihydroergotamine tartrate the drug is

\* We are indebted to Dr. Henze of the Sandoz Chemical Company for the supply of DHO 180, used in this study.

<sup>1</sup> Scherf, D., and Schlachman, M., *Am. J. Med. Science*, 1948, **216**, 673.

<sup>2</sup> Schlachman, M., and Scherf, D., *Am. Heart J.*, 1949, in press.

<sup>3</sup> Bluntschli, H. J., and Goetz, R. H., *Am. Heart J.*, 1948, **35**, 873.

<sup>4</sup> Kappert, A., *Helvetica Med. Acta*, 1949, Suppl. 22.

not contraindicated in patients with coronary disease.

**Summary.** The intravenous administration of 0.5 mg of dihydroergocornine to 24 patients with left ventricular hypertrophy caused no electrocardiographic changes indicative of coronary vasoconstriction. Although 6 of the 24 patients suffered from anginal pain and

many more certainly had coronary disease, no angina pectoris followed the injection.

This particular alkaloid of ergot seems to be a safe drug in patients with coronary artery disease.

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### 17211. Cultivation of Pseudorabies Virus in the Yolk Sac of the Developing Chick Embryo.

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Successful cultivation of pseudorabies on the chorioallantoic membrane of embryonated eggs has been achieved by a number of workers,<sup>1-6</sup> several of whom propagated the virus in serial transfer for more than 50 passages. They noted that the titer of virus increased with continued passage, ability to kill embryos was gained and a lengthened incubation period for laboratory animals was acquired. The virus, however, invariably produced fatal infection. Although no reports were found that related to the cultivation of pseudorabies virus by the yolk sac method of Cox,<sup>7</sup> the attenuation of rinderpest by Jenkins and Shope<sup>8</sup> and of Newcastle virus by Beach<sup>9</sup> prompted the hope that cultivation in this manner might modify pseudorabies virus also.

In both the rinderpest and Newcastle studies attention was centered on virus obtained from fluids, chorioallantoic membranes and embryos while titers attained in yolk sacs were not mentioned specifically. Indeed, other than for rickettsial and psittacoid agents, no information could be found that covered the growth of the smaller viruses in the yolk sac. In the work that follows attention was focused on growth of pseudorabies in the yolk sac membranes and the consequent effects on the virus.

**Technic.** The pseudorabies virus used throughout this work was a Hungarian strain sent to us through the courtesy of Dr. R. E. Shope in an infected rabbit brain preserved in glycerol. Part of the infected rabbit brain after washing was ground with sterile saline to make a homogeneous suspension and then 0.2 cc was inoculated intracerebrally into a rabbit. Two days later and immediately after death of the animal with typical symptoms of the disease, the brain was removed aseptically, weighed and ground in saline with a glass grinder to make a 10% suspension. The suspension was distributed in vials, sealed, quickly frozen and stored under dry ice refrigeration. Virus stored under such conditions was used to inoculate eggs in the initial passage. In subsequent passages 10% suspensions of fresh yolk sacs, prepared as for the rabbit brain, were used except in the first

<sup>1</sup> Mesrobian, I., *C. R. Soc. Biol.*, 1938, 127, 1183.

<sup>2</sup> Bedenski, G., and Bruckner, I., *C. R. Soc. Biol.*, 1938, 129, 406.

<sup>3</sup> Burnet, F. M., Lush, D., and Jackson, A. V., *Austral. J. Exp. Biol. and Med. Sc.*, 1939, 17, 35.

<sup>4</sup> Glover, R. E., *Brit. J. Exp. Path.*, 1939, 20, 150.

<sup>5</sup> Morril, C. C., and Graham, R., *Am. J. Vet. Res.*, 1941, 2, 35.

<sup>6</sup> Bang, F. B., *J. Exp. Med.*, 1942, 76, 263.

<sup>7</sup> Cox, H. R., *Pub. Hlth. Rep.*, 1939, 53, 2241.

<sup>8</sup> Jenkins, Dubois L., and Shope, R. E., *Am. J. Vet. Res.*, 1946, 7, 174.

<sup>9</sup> Beach, J. R., Bankowski, R. A., and Quortrup, E. R., *Cornell Vet.*, 1948, 38, 341.

TABLE I. (continued).  
Changes of Blood Pressure and Electrocardiogram Due to Dihydroergocorinine.

No.	Age	Sex	Diagnosis	Blood pressure			Electrocardiogram	
				Initial	5 min.	30 min.	Before	After
14*	57	M	Hypertensive cardiovascular Old cerebrovascular accident with slight hemiparesis	228/118	198/104	186/102	Left ventricular hypertrophy	" "
15	76	M	Hypertensive cardiovascular	228/94	194/80	204/92	" "	" "
16	82	M	Hypertensive cardiovascular Cerebrovascular accident	186/112	144/86	134/88	" "	" "
17	47	M	Essential hypertension	190/144	166/134	172/140	" "	" "
18	74	M	Hypertensive cardiovascular Congestive heart failure	192/104	164/84	156/94	" " Auricular fibrillation, myocardial damage	" "
19	72	F	Hypertensive cardiovascular Osteoarthritis	214/76	194/78	178/72	Left ventricular hypertrophy	" "
20*	58	F	Hypertensive cardiovascular Osteoarthritis, obesity	174/90	152/86	166/84	" "	" "
21	82	F	Hypertensive cardiovascular Arteriosclerotic cardiov. Possible thoracic aortic aneurysm	232/106	194/94	170/98	" "	" "
22*	75	F	Generalized arteriosclerosis Coronary sclerosis	160/80	150/72	128/64	" "	" "
23*	67	M	Coronary thrombosis (antero- lateral wall infarction)	140/86	130/80	118/80	" "	" "
24	80	M	Hypertensive cardiovascular Arteriosclerotic cardiov.	154/56	136/52	126/54	" " Ventricular extrasystoles	" "

\* History of anginal pain.

TABLE III.  
Distribution of Pseudorabies Virus in Eggs Inoculated into Yolk Sac.

Material used	Yolk sac passage	Dilutions				
		10-3	10-4	10-5	10-6	10-7
Yolk sac	1	0	+	+	+	0
	14	0	+	+	+	—
	50	0	0	+	+	—
Chorioallantoic membrane	1	0	+	+	—	0
	14	0	+	+	—	0
	50	0	+	+	—	—
Chorioallantoic fluid	1	+	—	—	0	0
	14	0	+	+	+	—
	50	0	0	+	—	—
Embryo	1	+	—	—	0	0
	14	0	+	+	—	0
	50	0	+	+	—	—

+ Virus shown to be present when inoculated intracerebrally into guinea pigs.

— Virus not present.

0 Not done.

In attempts to cultivate pseudorabies virus in the yolk sac, 9 fertile hens' eggs that had been embryonated for 6 days were inoculated in each passage and 3 eggs were left uninoculated as a test of incubator conditions. After inoculation, all eggs were incubated at 37°C and thereafter candled twice daily to record any changes in the embryos. Immediately after death of the embryos, the amount of virus was determined in the following manner. Tenfold dilutions were made from culturally sterile 10% suspensions in chilled saline and held in iced water from the time of preparation to completion of inoculations. From each dilution, 0.2 cc was inoculated intracerebrally into each of 1 or more rabbits and guinea pigs, 1 cc subcutaneously into each of 1 or more rabbits or guinea pigs, 0.05 cc intracerebrally into each of a group of 3 or 12 mice, and 0.5 cc subcutaneously into each of a group of 3 or 6 mice. Animals were observed twice daily and signs of illness recorded. In animals that showed no signs of illness, it was found that the first inoculation had conferred no immunity.

*Transfer of virus in serial passage.* After 5, 12, 26, 40 and 50 serial passages, tests were made for the presence and content of virus in the yolk sac. These results in comparison with the virus passed in rabbits are shown in Tables I and II.

As can be seen in Tables I and II, it ap-

peared that the virus was maintained in the yolk sac in serial transfer for 50 passages and that the titer remained unchanged or perhaps increased. As tested by intracerebral inoculation in mice, serum that neutralized a hundred infective doses of the virus passed in rabbits likewise neutralized a similar amount of virus transferred in eggs for 50 passages. This result permitted the conclusion that the death produced in rabbits, guinea pigs and mice with suspensions of yolk sac from eggs after 50 serial transfers was due to pseudorabies virus and not another agent encountered in passage.

*Distribution of virus in the egg.* After showing that pseudorabies virus infected yolk sacs, it became of interest to determine further distribution of the virus in eggs inoculated in this manner. Each of a group of 12 eggs was inoculated with a 10% suspension of brains from infected rabbits, another with a 10% suspension of yolk sacs from infected eggs that had been transferred serially for 13 passages, and a third group with a 10% suspension of yolk sacs from infected eggs transferred for 49 passages. Eggs inoculated with virus passed in rabbits were harvested 3 days after inoculation, while those in the serial passages were harvested after 40 hours. From these eggs chorioallantoic fluids, yolk sacs, embryos and chorioallantoic membranes were obtained separately, and with the excep-



TABLE I.

Results in Experimental Animals Inoculated Intracerebrally with Various Dilutions of Pseudorabies Virus Cultivated in the Yolk Sac of Chick Embryos.

Test animals*	Yolk sac passage	Dilutions used†				
		10-3	10-4	10-5	10-6	10-7
Rabbits	0	—	1/1	2/3	0/2	—
	5	—	1/1	1/1	1/1	0/2
	12	—	1/1	1/1	1/1	0/1
	26	—	—	1/1	0/1	0/1
	40	—	2/2	1/1	0/1	—
	50	—	1/1	1/1	1/1	0/1
Guinea pigs	0	1/1	2/2	0/2	0/1	—
	5	1/1	3/3	3/3	0/2	—
	12	1/1	2/2	4/4	2/2	0/1
	26	1/1	1/1	1/1	0/1	0/1
	40	1/1	1/1	1/1	0/1	—
	50	1/1	1/1	1/1	1/1	0/1
Mice	0	3/3	12/12	2/6	0/3	—
	5	3/3	9/9	6/6	0/3	—
	12	3/3	6/6	9/9	5/6	1/3
	26	3/3	3/3	3/3	0/3	0/3
	40	—	3/3	3/3	0/3	—
	50	—	3/3	3/3	2/3	0/3

\* .2 cc of the virus dilution inoculated in each rabbit and guinea pig and .05 cc in each mouse.

† Denominator indicates number of animals used; numerator indicates animals that showed pseudorabies.

TABLE II.

Results in Experimental Animals Inoculated Subcutaneously with Various Dilutions of Pseudorabies Virus Cultivated in the Yolk Sac of Chick Embryos.

Test animals*	Yolk sac passage	Dilutions used†							
		10-1	10-2	10-3	10-4	10-5	10-6	10-7	10-8
Rabbits	0	—	—	—	2/2	2/2	0/1	—	—
	5	—	—	—	1/1	1/1	1/1	0/1	—
	12	—	—	—	1/1	1/1	1/1	1/1	0/1
	26	—	—	—	—	1/1	0/1	0/1	—
	40	—	—	1/1	1/1	1/1	0/1	—	—
	50	—	—	1/1	1/1	1/1	0/1	—	—
Guinea pigs	0	1/1	3/3	2/3	0/1	—	—	—	—
	5	—	2/2	3/3	3/3	0/1	—	—	—
	12	1/1	1/1	2/2	3/3	2/2	0/1	—	—
	26	1/1	1/1	1/1	0/1	0/1	0/1	0/1	—
	40	2/2	1/1	1/1	0/1	0/1	—	—	—
	50	2/2	1/1	1/1	0/1	0/1	—	—	—
Mice	0	—	3/3	2/3	0/6	0/3	0/3	—	—
	5	—	6/6	3/3	6/6	0/3	0/3	—	—
	12	—	3/3	3/3	6/6	0/3	0/3	—	—
	26	—	—	3/3	1/3	0/3	0/3	0/3	—
	40	3/3	3/3	3/3	0/3	—	—	—	—
	50	—	6/6	3/3	0/3	—	—	—	—

\* 1 cc of the virus dilution inoculated in each rabbit and guinea pig and 0.5 cc in each mouse.

† Denominator indicates number of animals used; numerator indicates animals that showed pseudorabies.

few passages where this material was stored for 48 hours at 34°F. This condition of storage was found not to affect the titer of

virus. In tests for sterility, samples from all inocula were placed on blood agar plates and blood agar slants. All showed no growth.

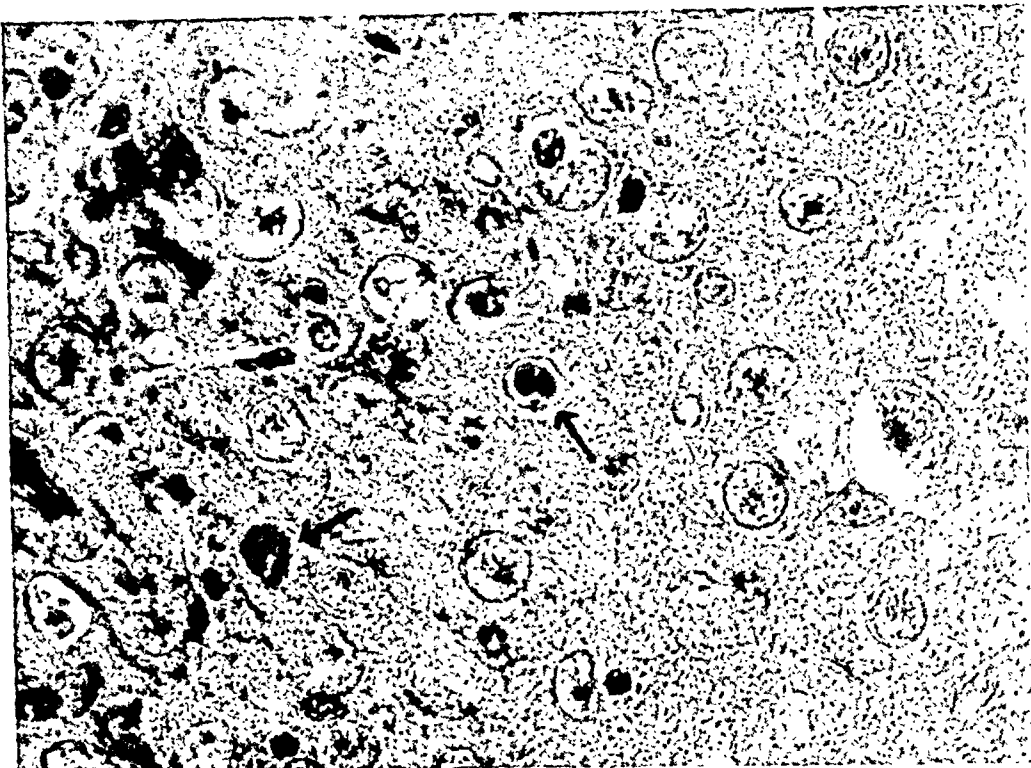


FIG. 3.

Brain from infected guinea pig inoculated intracerebrally with virus passed in rabbits. Arrow indicates inclusions in nuclei.

tion of chorioallantoic fluids, washed in 3 changes of saline, triturated and 10% suspensions made. Tenfold dilutions then were prepared from each suspension and tested in guinea pigs by intracerebral inoculations. The results are shown in Table III.

In Table III, it can be seen that the yolk sac always contained as much or more virus than other parts of infected eggs.

*Effect of virus transferred in eggs on animals.* In the first passage of pseudorabies virus into eggs, 60% of the embryos died 3 days after inoculation, in the 5th passage 80% of the embryos died within 48 hours, and in the 12th and subsequent passages all embryos died within a period of 40 hours.

In the rabbits, guinea pigs and mice inoculated both intracerebrally and subcutaneously with virus passed in rabbits and transferred 5, 12, 40 and 50 passages, comparisons were made of the effects produced by 10 to 100 lethal doses of virus. It was found that

with continued serial transfer of virus in eggs, the interval of time between inoculation and death lengthened and animals survived longer after showing visible signs of illness. Moreover, a marked change in the signs of experimental disease was observed in inoculated animals. When rabbits were inoculated with virus passed in rabbits subcutaneously, characteristic skin signs and lesions in the form of an intense pruritus at the site of inoculation invariably developed. The skin became denuded of hair, abraded and infiltrated with serosanguinous fluid. Virus transferred in the yolk sac for 5, for 12, for 26 and for 40 passages produced similar signs, while animals that received virus transferred for 50 passages failed to develop signs or lesions in the skin. Instead, leg weakness developed 2 to 4 days before they died. Guinea pigs showed changes similar to rabbits except that the characteristic signs and lesions disappeared when the virus had been transferred for 40

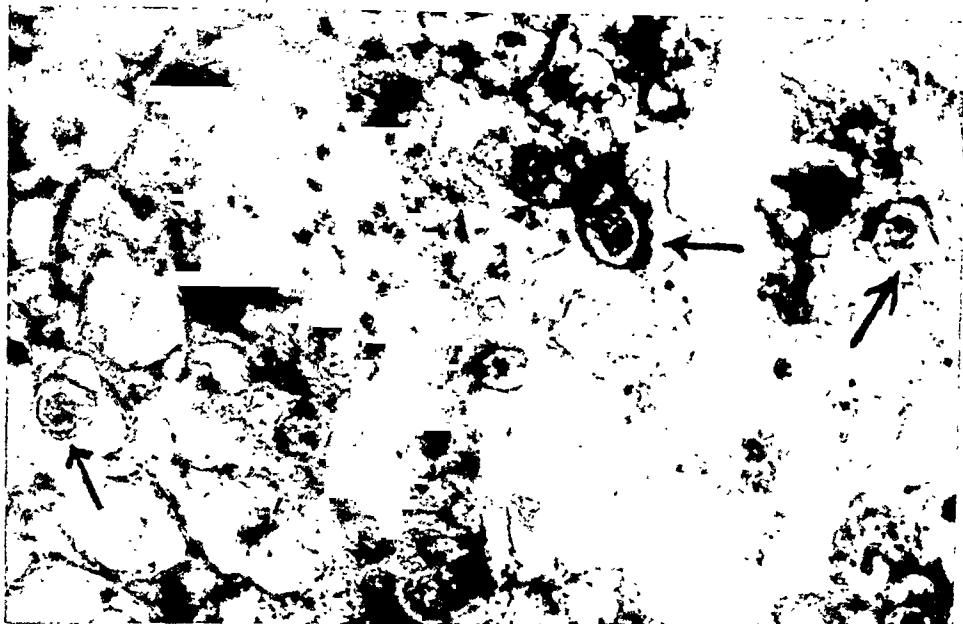


FIG. 1.

Yolk sac from an egg inoculated with virus transferred for 50 passages. Arrow indicates intranuclear inclusions.

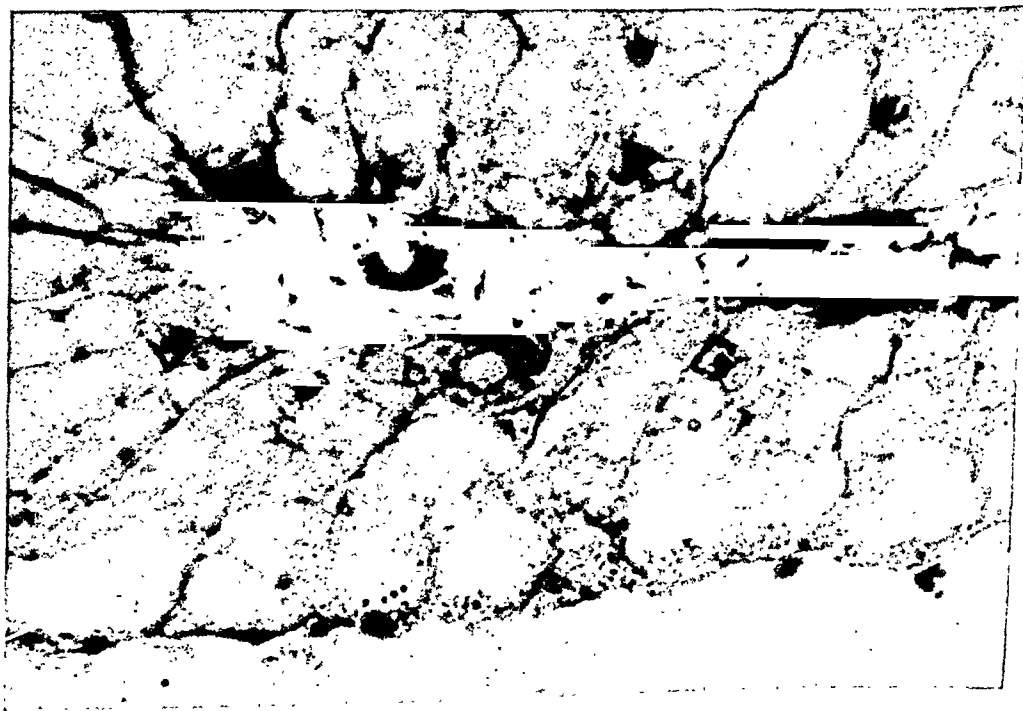


FIG. 2.

Yolk sac from an egg inoculated with saline, showing normal appearance of nuclei of yolk sac cells.

for 5 passages in eggs, acidophilic small granules that were accumulated around the nucleolus was the only change observed. In the yolk sacs of eggs inoculated with saline, no changes were seen (Fig. 2).

Brains from guinea pigs inoculated intracerebrally with the rabbit brain virus and virus that had been transferred for 50 passages in eggs were fixed in Zenker's or formalin fixative and stained as for the egg material. In some brains from guinea pigs inoculated either with virus passed in rabbits or virus transferred for 50 serial passages in eggs, slight congestion was the only change observed, while in others, leucocytic infiltration and marked congestion of the blood vessels of the meninges and the superficial part of the brain were seen. Most of the animals showed the superficial nerve cells in different stages of degeneration. Intranuclear inclusions similar to those observed in the cells of yolk sacs from infected eggs were found in some nerve cells (Fig. 3 and 4).

**Summary.** Using the yolk sac method, a strain of pseudorabies virus has been cultivated in eggs for 50 serial transfers. That the virus multiplied in the yolk sac was shown in 4 ways: (1) suspensions of yolk sacs from infected eggs that had been transferred either

for 5, 12, 26, 40 or 50 passages produced disease in rabbits, guinea pigs and mice; (2) the effects in animals of virus that had been transferred for 50 serial passages was neutralized by antiserum that neutralized virus passed in rabbits; (3) the yolk sac contained as much or more virus than either the chorioallantoic membranes, chorioallantoic fluid or embryos; (4) the virus produced characteristic intranuclear inclusions in the yolk sac cells like those seen in cells from infected animals.

With continued transfer of pseudorabies virus in eggs, embryos died 40 hours after inoculation while in the initial passage only 60% of the embryos died after 3 days. The capacity to invade certain parts of the egg such as the chorioallantoic fluids and embryos increased and more extensive changes were produced in the embryo. Continued passage also altered the effects on rabbits, guinea pigs and mice. The period of time from inoculation until death of animals lengthened, the local pruritus produced by subcutaneous inoculation of virus passed in rabbits no longer occurred and infected animals lived longer after showing signs of illness.

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## 17212. Antagonism of Choline and Heparin *In vivo* and *In vitro*.\*

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(With the technical assistance of Mrs. Marie T. Wasson.)

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Cabezas and Honorato<sup>1</sup> reported that heparin was inactivated by choline chloride. Various dilutions of heparin, choline and combinations of the 2 were added to fresh and 24-hour preserved plasma. The influence

upon the prothrombin time was determined by the one stage Quick method.<sup>2</sup> These investigators did not draw final conclusions as to the mechanism of this effect, but suggested that there may be a chemical union of the choline with heparin, seroalbumin or albumin X.<sup>3</sup>

The annulation of heparin with prota-

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\* This work was supported, in part, by the American Cancer Society on recommendation of the Committee on Growth of the National Research Council, and American Cancer Society Grant INSTR-23A.

† Rosalie B. Hite Postdoctoral Fellow.

<sup>1</sup> Cabezas, A., and Honorato, R., *Comunic. Society Biol. Santiago, Chile*, 1944, 2, 26.

<sup>2</sup> Quick, A. J., *J. Am. Med. Assn.*, 1940, 119, 118.

<sup>3</sup> Quick, A. J., *The Hemorrhagic Diseases and the Physiology of Hemostasis*. Thomas, Editor, 1942.

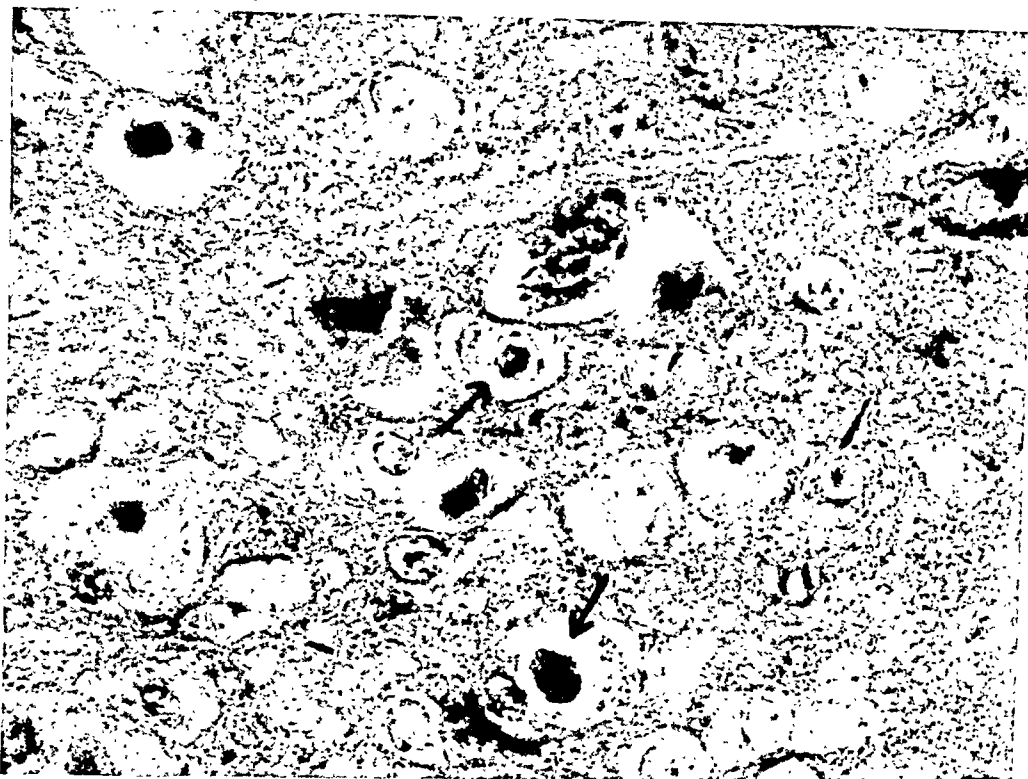


FIG. 4.

Brain from guinea pig inoculated intracerebrally with virus transferred for 50 serial passages in eggs. Arrow indicates inclusions.

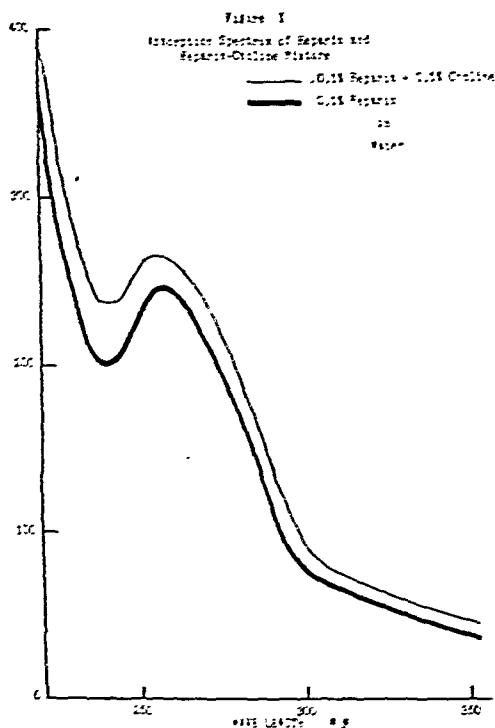
passages in eggs. The animals then died with signs simulating those in guinea pigs inoculated intracerebrally. Mice that were inoculated subcutaneously showed paralytic-like signs when given virus transferred for 40 passages in eggs. Pruritus was not seen in mice even when inoculated with virus passed in rabbits.

*Pathology.* Embryos from the initial passage and each subsequent one were examined for lesions. In the first few passages, hemorrhages in the skin were seen over the brain region, but later hemorrhages extended over the entire surface of the embryos.

Brains of embryos from eggs inoculated with virus transferred for either 15 or 50 serial passages in eggs were prepared in Zenker's fixative and stained with hematoxylin and eosin and by Wilhite's method. Examination showed congestion of the superficial blood vessels with leucocytic infiltration throughout the brain. The neurons and neuro-

glial cells showed various stages of degeneration. Some of the mesothelial cells of the membranes covering the brain showed intranuclear inclusions in the form of homogeneous acidophilic material filling part or all of the nucleus. In brains of embryos from eggs inoculated with either rabbit brain virus or virus transferred for 5 passages, these changes were less pronounced and in some, only a slight congestion of the superficial blood vessels was found. Brains of embryos from eggs inoculated with saline showed no changes.

The most important changes seen in the cells of yolk sacs from eggs inoculated with virus transferred 50 passages were intranuclear inclusions (Fig. 1) that appeared as acidophilic homogeneous material filling most of the nucleus when completely formed. Margination of the chromatin along the inner surface of the nuclear membrane was seen. In the yolk sac cells of eggs inoculated with virus passed in rabbits and virus transferred



intravenously in an ear vein weekly with solutions of heparin and choline in the following ways:

- 1) 100 IU kg heparin;
- 2) 10 mg kg 2% choline chloride in saline;
- 3) 100 U kg heparin followed immediately by 10 mg kg choline;
- 4) 10 mg kg choline followed in 5 min. by 100 U kg heparin;
- 5) 100 U kg heparin followed in 5 min. by 10 mg kg of choline.

Blood was obtained by cardiocentesis with a 22 gauge needle. A specimen was drawn prior to and 10, 20, 30 and 60 minutes after injection. Coagulation times were done by a modification of the Lee-White method.<sup>19</sup> Blood in the amount of 0.5 cc was added to a 10 by 75 mm glass tube which had been

rinsed with saline. The tube was tilted every 30 seconds and was kept at a temperature from 32 to 35°C. The end point was taken as the time the tube could be tilted vertically and the clot was solid. The prothrombin time was done by the one stage Quick method on whole plasma, and 0.9 cc of blood was added to 0.1 cc of 0.1 M sodium oxalate.

**Results.** Normal coagulation time varied between 3½ and 14 minutes. The choline alone had no remarkable effect on the blood coagulation other than possibly slightly accelerating it. Heparin alone, and heparin and choline in the various combinations, produced similar results.

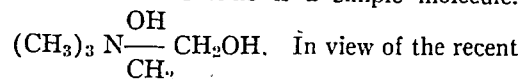
The normal prothrombin times varied between 5.7 and 7.8 seconds and were not affected by the choline, whereas, they were slightly prolonged from 0.7 to 2.4 seconds after heparin alone, and 18.5 seconds after the heparin-choline injections. All prothrombin times were done in duplicate. If more than one second difference was obtained a third test was performed.

**Interpretation.** By means of the coagulation time and prothrombin time *in vivo*, choline has been shown to have no antagonistic influence on the heparin effect. One interesting observation is that choline alone consistently accelerated coagulation. This group of rabbits is too small, however, to draw definite conclusions as to the effect of choline on coagulation. Therefore, this is being studied further.

**Summary.** The previously reported antagonism of choline and heparin was studied *in vitro* and *in vivo*. When choline and heparin were mixed in whole plasma and the prothrombin time determined, the choline had no effect on the action of heparin. Choline alone slightly prolonged the prothrombin time. Choline and heparin do not combine chemically as shown by spectrophotometric determination. *In vivo*, choline has no effect on the action of heparin.

<sup>19</sup> Lee, R. L., and White, P. D., *Am. J. Med. Sc.*, 1913, 145, 495.

mine or toluidine blue is well established.<sup>4-6</sup> These latter compounds are complex molecules whereas choline is a simple molecule.



work by Allen, Jacobson, Smith *et al.*, on the hemorrhagic syndrome produced by exposure to heavy doses of X-irradiation or nitrogen mustard,<sup>7,8</sup> the inactivation of heparin by choline becomes of considerable physiological and clinical interest. They have shown that this syndrome is due to an increase in the heparin or a heparin-like compound in the blood. Through 2 approaches *in vitro* and one approach *in vivo*, we have attempted to elucidate this problem.

*Influence of choline and heparin upon the prothrombin time.* A portion of Cabezas and Honorato's work was repeated as accurately as possible. The prothrombin time, Quick method,<sup>2</sup> was carried out on fresh whole human plasma. Choline chloride (2% and 4%) (Merck) in physiological saline was used in various volumes. Commercial heparin, 10 mg/cc (Roche-Organon) was diluted with physiological saline so that 0.025 cc equaled approximately 0.4 International Units and 0.025 cc equaled approximately 0.2 International Units. The commercial heparin contains approximately 110 International Units per mg. Rabbit brain thromboplastin (Maltine Co.) was used. The choline-heparin mixtures were not incubated.

*Results.* The prothrombin times on the control plasmas averaged 14.5 seconds; 0.2 U heparin added to the plasma showed an increase in the prothrombin time, averaging 4.9 seconds; 0.4 U heparin increased the prothrombin time an average of 13.7 seconds. Fifty  $\gamma$ , 2% choline, increased the prothrom-

bin time an average of 1.0 seconds; 100  $\gamma$ , 2% choline, increased the prothrombin time an average of 3.1 seconds; 100  $\gamma$ , 4% choline, increased the prothrombin time an average of 2.9 seconds. Mixtures of heparin and choline in various concentrations and volumes added to the plasma always produced an increase in the prothrombin time over that of the heparin alone.

*Interpretation.* Our data illustrated no inactivation of the heparin by the choline. The slight increase in prothrombin time demonstrates the *in vitro* anticoagulant influence of choline. This confirms the observations of Zunz and LaBarre.<sup>9</sup>

When one analyzes Cabezas and Honorato's results, it is observed that the control prothrombin times were prolonged in most experiments. This is evidence of relatively inactive thromboplastin.

*Absence of a chemical union of heparin and choline as measured by spectrophotometric determination.* By the use of the Beckman spectrophotometer the absorption spectrum of a 0.1% solution of powdered heparin (Connaught 110 U/mg) dissolved in distilled water was determined. The peak of the absorption curve was found to be 265 m $\mu$ . Choline chloride in a 0.1% solution has no typical absorption bands. The two solutions were mixed in equal parts and the absorption curve was determined immediately, and after 6 hours of incubation.

*Results.* As may be seen in Fig. 1, there was no change in the absorption spectrum after heparin and choline were mixed.

*Interpretation.* If choline were combined with heparin there would be a definite shift in the spectrum of the choline-heparin mixture from that of the heparin alone. This demonstrates quite conclusively that heparin and choline do not enter into a chemical combination.

*In vivo influence of heparin-choline on coagulation and prothrombin time.* A series of experiments were designed to give *in vivo* evidence concerning the action of these substances on the coagulation mechanism. Three rabbits, each weighing 2.0 kg, were injected

<sup>4</sup> Chargaff, L., and Olson, B., *J. Biol. Chem.*, 1937, **122**, 153.

<sup>5</sup> Wilander, O., *Skand. Arch. and Physiol.*, 1938, **81**, Suppl. XV.

<sup>6</sup> Jorpes, J. E., *Heparin in the Treatment of Thrombosis*, Oxford University Press, 1946.

<sup>7</sup> Allen, J. G., and Jacobson, L. O., *Science*, 1947, **105**, 388.

<sup>8</sup> Smith, T. R., Jacobson, L. O., Spurr, C. L., Allen, T. G., and Block, M. H., *Science*, 1948, **107**, 474.

<sup>9</sup> Zunz, E., and LaBarre, J., *Compt. Rend. Biol.*, 1924, **90**, 655.

with the patients with myasthenia gravis for one week. Thereafter, the patients were administered intramuscularly adrenocorticotrophic hormone in amounts of 20 mg every 6 hours for 5 days. Tests were performed during the administration of the hormone, for 4 days thereafter, and at biweekly intervals for another 12 weeks. The following tests were performed.

*Electromyography.* Muscle action potential records were taken during repetitive indirect percutaneous stimulation of the ulnar nerve by 10 and 30 pulses per second for 30 seconds, with a stimulus of supramaximal intensity and of one millisecond duration. The method was described by Harvey and Masland<sup>13</sup> and Torda and Wolff.<sup>12</sup> The records were taken daily at the same time of the day, 3 hours after administration of neostigmine bromide during the control period and the period of administration of the hormone, and from 6 to 15 hours after administration of neostigmine bromide thereafter.

*Myography.* Muscle function was also tested by an ergograph immediately after completion of the electromyograms. The ergograph consisted of a heavy spring attached to an isotonic lever writing on a kymograph. The spring was stretched 2.5 cm once a second by the index and middle finger of the right hand exerting a tension of 15 kg.

*Acetylcholine synthesis.* Acetylcholine synthesis in the presence of the blood serum was studied following the method described by Torda and Wolff.<sup>2</sup> The method consists of incubation of a tissue containing choline acetylase with blood serum and determination of the amount of acetylcholine formed during the period of incubation. The amount of acetylcholine synthesized in the presence of serum from healthy controls averaged  $2.08 \mu\text{g} \pm 7\%$  per 100 mg tissue containing the enzyme.

*Results.* During the 5 days of administration of ACTH all patients experienced a gradually increasing disability lasting until the second day after the completion of the injections. Thereafter, the patients exhibited increased well being, they began to reduce the daily intake of neostigmine bromide spontaneously (H.L. from 300 mg to 45 mg; M.Y.

from 150 mg to 15 mg; A.S. from 112 mg to 15 mg; R.G. from 180 mg to 90 mg; and J.R. from 45 mg to 15 mg) and omitted the other medications. A partial remission of the symptoms occurred in all instances manifesting itself in marked decrease of muscle weakness, the easy fatiguability, and the anorexia. The ptosis of the eyelids, the weakness and easy fatiguability of the muscles of palate, tongue, face, deglutition, arms, and legs became less evident. However, the remission was incomplete, the muscle groups most severely involved in each patient showed only a partial recovery, as is to be expected after a muscle dysfunction of several years duration. Though not clearly definable, it is probable that there is some dwindling of the improvement in muscle function with the lapse of time. This incomplete remission persisted from the completion of administration of the hormone to the writing of this report (approximately 3 months).

*Electromyography.* Healthy subjects maintained the muscle action potential unaltered during repetitive indirect stimulation with 10 pulses per second for 30 seconds (Fig. 1A) and nearly unaltered (average decrease of 12%) during stimulation with 30 pulses per second for 30 seconds.<sup>12,13</sup> Before administration of the hormone patients H.L., M.Y., A.S., and R.G. exhibited the known decline of the amplitude of muscle action potential during repetitive indirect stimulation. The decline of the amplitude exceeded in all instances 35% on stimulation with 10 pulses per second for 30 seconds and 55% on stimulation with 10 pulses per second for 30 seconds (Fig. 1B-1E). During the period of administration of the hormone the abnormalities noted in the electromyograms of the patients gradually decreased. After completion of administration of the hormone and while on reduced medication the patients were so altered as to maintain the amplitude of action potential during repetitive indirect stimulation in a manner resembling that of healthy subjects. Patient J.R. had before and after administration of the hormone a normal electromyogram (Fig. 1F) as commonly occurs in patients mildly to moderately ill with myasthenia gravis. The electromyograms re-



# 17213. Effects of Adrenocorticotrophic Hormone on Neuro-Muscular Function in Patients with Myasthenia Gravis.\*

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The adrenocorticotrophic hormone of the pituitary gland has been administered to patients with myasthenia gravis mainly on the basis of the following observations and inferences: 1) the immediate cause of the symptoms of myasthenia gravis is a decrease of acetylcholine synthesis;<sup>1-3</sup> 2) administration of the adrenocorticotrophic hormone increases acetylcholine synthesis *in vivo*;<sup>4</sup> 3) increase of the lymphatic tissue (round-cell infiltration of various organs, mainly striated muscle<sup>5</sup> and "hyperfunctioning" thymus<sup>6</sup> have been found in patients with myasthenia gravis. Tissue fractionation studies<sup>7,8</sup> have shown that one of the sources of the substances that inhibit acetylcholine synthesis is the thymus. Administration of the adrenocorticotrophic hormone induces reduction in the mass of the thymus and the lymphatic tissue;<sup>9,10</sup> 4) removal of the pituitary gland in rats induces changes in the electromy-

gram<sup>11</sup> that closely resemble the abnormalities noted in patients with myasthenia gravis;<sup>12,13</sup> 5) the pituitary gland of several patients who died of myasthenia gravis showed accumulation of an eosinophilic colloid material suggesting altered function of the gland.<sup>14-18</sup>

This report aims to illuminate the nature of myasthenia gravis by a further analysis of its phenomenology. Therapeutic implications are outside its scope.

**Material.** Five patients with myasthenia gravis were women aged 24 (H.L.), 29 (J.R.), 31 (M.Y.), 37 (A.S.), and 45 (R.G.) years who had had myasthenia gravis for 4, 17, 10, 13, and 9 years, respectively. During the 3 years before this special study begun the patients experienced minor transient fluctuations but no long lasting or significant changes in their clinical states. They received a total of 300, 45, 180, 112, and 150 mg of neostigmine bromide a day, distributed over the waking hours, taken in from 3 to 6 hourly intervals. H.L. received also 75 mg of ephedrine sulfate, 3 g of potassium chloride, and 0.39 g of guanidine hydrochloride a day; R.G. also received 25 mg of ephedrine sulfate and 3 g of potassium chloride a day. The patients had had, in different degree of severity anorexia, general weakness, ptosis of the eyelids, weakness and easy fatigability of the muscles of the palate, tongue, face, deglutition, arms, and legs.

**Method.** Various tests were performed

\* Published with permission of the Chief Medical Director, Departments of Medicine and Surgery, Veterans Administration, who assumes no responsibility for the opinions expressed or conclusions drawn by the authors.

<sup>1</sup> Torda, C., and Wolff, H. G., *Science*, 1943, **98**, 224.

<sup>2</sup> Torda, C., and Wolff, H. G., *J. Clin. Invest.*, 1944, **23**, 649.

<sup>3</sup> Torda, C., and Wolff, H. G., *Science*, 1944, **100**, 200.

<sup>4</sup> Torda, C., and Wolff, H. G., *Proc. Soc. Exp. Biol. and Med.*, 1944, **57**, 137.

<sup>5</sup> Weigert, C., *Neurol. Centralbl.*, 1901, **20**, 597.

<sup>6</sup> Sloan, H. H., Jr., *Surgery*, 1943, **13**, 154.

<sup>7</sup> Torda, C., and Wolff, H. G., *Proc. Soc. Exp. Biol. and Med.*, 1944, **57**, 69.

<sup>8</sup> Torda, C., and Wolff, H. G., *Am. J. Physiol.*, 1947, **148**, 417.

<sup>9</sup> Dougherty, T. F., and White, A., *Proc. Soc. Exp. Biol. and Med.*, 1943, **53**, 132.

<sup>10</sup> Simpson, M. E., Li, C. H., Reinhardt, W. O., and Evans, H. M., *Proc. Soc. Exp. Biol. and Med.*, 1943, **54**, 135.

<sup>11</sup> Torda, C., and Wolff, H. G., *Am. J. Physiol.*, 1949, **156**, 274.

<sup>12</sup> Torda, C., and Wolff, H. G., *Arch. Int. Med.*, 1947, **60**, 68.

<sup>13</sup> Harvey, A. M., and Masland, R. L., *Bull. Johns Hopkins Hosp.*, 1941, **69**, 1.

<sup>14</sup> Buzzard, E. F., *Brain*, 1905, **28**, 438.

<sup>15</sup> Tilney, F., *Neurographs*, 1907, **1**, 20.

<sup>16</sup> Marinresco, G., *Semaine Med.*, 1908, **28**, 421.

<sup>17</sup> Cavallero, C., *Tumori*, Ser. 2, 1944, **20**, 127.

<sup>18</sup> Torda, C., and Wolff, H. G., in press.

sion of the symptoms of myasthenia gravis in a moderately ill woman after administration of the hormone was reported by Soffer and collaborators<sup>20</sup> without application of objective testing procedures. The transitory impairment observed during the period of administration of the hormone was also observed by Hellman.<sup>21</sup> The increased general disability during the administration of the hormone may be due to changes in electrolyte distribution,<sup>22</sup> and in various metabolic processes (increase of secretion of some steroid hormone,<sup>22-26</sup> adverse effect on carbohydrate metabolism<sup>25</sup> and decrease of glutathione content of blood.<sup>23</sup>) These processes apparently offset the gradually improving function of the neuromuscular apparatus *per se*.<sup>19</sup>

The above investigations indicate that administration of the adrenocorticotrophic hormone induces changes suggesting the begin-

nings of an incomplete remission in patients with myasthenia gravis. If the view suggested by this laboratory<sup>1-3</sup> be valid, *i.e.*, that the immediate cause of the symptoms of patients with myasthenia gravis is a decrease in the synthesis of acetylcholine, then the observation that administration of the hormone increases the synthesis of acetylcholine<sup>4</sup> becomes extremely pertinent to an understanding of the apparent remission of symptoms observed in patients with myasthenia gravis after administration of the adrenocorticotrophic hormone. Because of its long lasting effects, it is inferred that the adrenocorticotrophic hormone acts upon some basic regulatory mechanism.

*Summary.* 1. Five patients moderately to severely ill with myasthenia gravis received 400 mg adrenocorticotrophic hormone given in amounts of 20 mg every 6 hours.

2. Changes that may indicate the beginnings of an incomplete remission occurred after completion of the injections. These changes consisted of decrease of the symptoms and outward manifestations of muscle dysfunction, disappearance of the abnormalities noted in the electromyogram, increased work performance on the ergograph, and increase to normal of the ability of serum to support acetylcholine synthesis. The incomplete remission is long lasting.

The authors wish to express their gratitude to John R. Mote, Medical Director of Armour Laboratories, Inc., for the generous supply of the adrenocorticotrophic hormone of the pituitary gland.

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<sup>20</sup> Soffer, I. J., Gabrilove, J. L., Laqueur, H. P., Volterra, M., Jacobs, M. D., and Sussman, M. L., *J. Mount Sinai Hosp.*, 1948, 15, 73.

<sup>21</sup> Hellman, L., *Fed. Proc.*, 1949, 8, 72.

<sup>22</sup> Conn, J. W., Louis, L. H., and Johnston, M. W., *J. Lab. Clin. Med.*, 1949, 34, 255.

<sup>23</sup> Forsham, P. H., Thorn, G. W., Prunty, F. T. C., and Hills, A. G., *J. Clin. Endocr.*, 1949, 8, 15.

<sup>24</sup> Thorn, G. W., Prunty, F. T. G., and Forsham, P. H., *J. Clin. Endocr.*, 1947, 7, 459.

<sup>25</sup> Conferences of Macy Foundation, Metabolic Aspects of Convalescence, 16 Meeting, Oct. 27, 1947; 17 Meeting, Spring, 1948.

<sup>26</sup> Mason, H. L., Power, M. H., Ryncarson, E. H., Letizia, C., Ciaramelli, M. D., Li, C. H., and Evans, H. M., *J. Clin. Endocr.*, 1948, 8, 1.

## 17214. Alkaline and Acid Phosphatase Activity of the Embryonic Chick Retina.

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Evidence has been accumulating which indicates that the phosphomonoesterases may

<sup>1</sup> Moog, F., *J. Cell. and Comp. Physiol.*, 1946, 28, 197.

be associated in some way with the differentiation of embryonic tissue. Moog<sup>1</sup> has demonstrated that the common alkaline and acid phosphomonoesterase activity increases

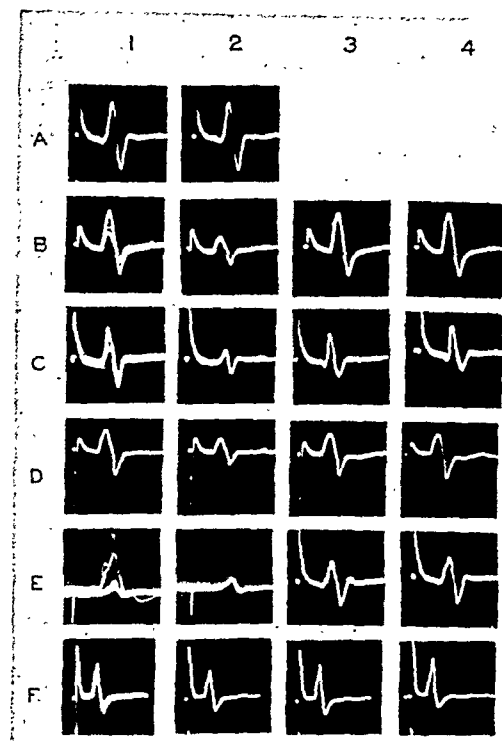


Fig. 1.

Effect of adrenocorticotrophic hormone on muscle action potential during repetitive indirect percutaneous stimulation of the ulnar nerve with 10 pulses per second.

Column 1 represents action potential records taken during the first few pulses of a 30 second stimulation period. Column 2 represents records taken at the end of the 30 second stimulation period. Column 3 represents action potential records taken during the first few pulses of a 30 second stimulation period the third day after completion of the series of administrations of the hormone. Column 4 represents records taken at the end of the 30 second stimulation period. Records in column 1 are comparable with records in column 2 of the same line and records in column 3 are comparable with those of column 4 of the same line since the position of the electrodes remained unchanged during the 30 second stimulation period. Records in column 3 are not comparable with those in column 1 of the same line since the recording electrodes could not be placed in exactly the same position on successive days. In all records the sweep circuit of the oscilloscope was synchronized with the stimulator so that successive stimuli and muscle action potentials were superimposed on the screen of the cathode ray tube. Group A contains records taken from a healthy control; group B records from patient H.L., group C records from M.Y., group D records from A.S., group E records from R.G., and group F records from J.R.

mained normal up to the writing of the pres-

ent report (approximately 3 months).

**Myography.** The 10 control women stretched the spring on the average 150 times before occurrence of fatigue. Before administration of the hormone the patients with myasthenia gravis the average number of contractions (10 exper.) before occurrence of fatigue were 31 (R.G.); 44 (A.S.); 47 (M.Y.); 75 (H.L.), and 110 (J.R.). During the period of administration of the hormone the number of contractions slightly and gradually increased. After completion of administration of the hormone the work performance continued to increase and reached the fifth day the maximum. This was 105 (R.G.); 120 (A.S.); 180 (M.Y.); 250 (H.L.); and 175 (J.R.). Although this test involves motivation, such sudden and dramatic increase in performance suggests an improvement of the function of the neuromuscular system *per se*. The improved work performance persisted for the past 3 months.

**Acetylcholine synthesis.** In the presence of serum of the patients with myasthenia gravis the synthesis of acetylcholine as compared to healthy control subjects decreased by 55 (R.G.); 50 (H.L.); 42 (M.Y.); 40 (A.S.), and 25 (J.R.) %. In the presence of blood serum taken the third day after completion of administration of the hormone and biweekly thereafter during the past three months the ability of serum to support acetylcholine synthesis approximated normal values. Decreased acetylcholine synthesis in the presence of serum is specific for myasthenia gravis. The more severe the myasthenia gravis the less well the serum supports the activity of choline acetylase.

**Comment.** Administration of adrenocorticotrophic hormone to patients with myasthenia gravis was first begun by Torda and Wolff.<sup>4,19</sup> Beginnings of remission were observed in the 2 patients treated in the New York Hospital. Because satisfactory objective procedures testing neuromuscular function *per se* had not been elaborated in this laboratory at the time inferences concerning the effect of the hormone on patients with myasthenia gravis were deferred. A remis-

<sup>19</sup> Torda, C., and Wolff, H. G., unpublished data, also-Records of New York Hospital.

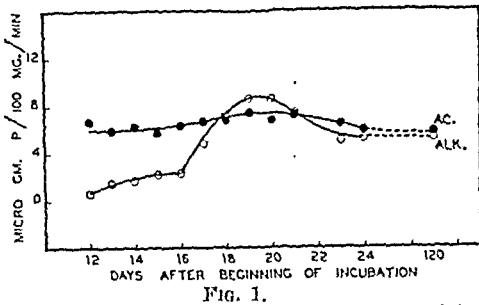


Fig. 1.  
Alkaline (ALK) and acid phosphatase activity of the developing chick embryo retina.

phosphate liberated per 100 mg of fresh tissue per minute.

Fig. 1 depicts graphically the data obtained for both the alkaline (ALK) and acid (AC) phosphatase activity of whole homogenates of chick embryo retinas beginning with the 12th day of incubation and ending 3 days after hatching. The points on the curves represent means of data obtained from 3 separate series of embryos, incubated under identical conditions. It will be noted that the alkaline phosphatase activity at 12 days is almost negligible but rises gradually up to 16 days of incubation. Beginning with 16 days the activity increases rapidly up to 19 days where it reaches a maximum which is maintained through the 20th day after which it begins to fall at a rate comparable to the rise. However, about 3 days after hatching it levels off and maintains a rather constant rate.

The activity of the acid phosphatase, on the other hand, is relatively high at 12 days of incubation and does not appear to show any rise until about the 15th day. At this time there is a very small rise up to 19 days when it also decreases and eventually levels off at about 24 days. The broken lines on the graph designate the level of enzyme activity between the 24th and 120th day. Weekly analyses made between these ages showed no appreciable fluctuation in the enzyme activity and it is assumed that no further change took place.

It is interesting to note that the peak of both alkaline and acid phosphatase activity is reached on about the 19th-20th day of incubation. That the retina is differentiated at this time was demonstrated by the author,<sup>5</sup> when it was shown that 18 days after the

beginning of incubation is the earliest stage in the development of the eye at which the pupillary constrictor reflex could be elicited by light stimuli.

Since these 2 events, namely completion of differentiation and development of maximum phosphatase activity correspond so closely it would appear that these enzymes may be concerned in some way with the morphogenesis of the retinal elements. These results are of interest therefore in the light of the findings of Moog, who demonstrated strikingly similar increases in both the alkaline and acid phosphatase activity of homogenates of whole chick embryos between the 2nd and 12th day of incubation during which time it was assumed that the tissues were undergoing differentiation. Thus the chick retina, which is known to differentiate between the 12th day of incubation and hatching time, lends further support to the concept that certain phosphomonoesterases may enter into the histochemistry of differentiation of embryonic tissues.

*Summary and conclusion.* 1. The alkaline and acid phosphatase activity was determined on whole homogenates of embryonic chick retina from the 12th day of incubation until 3 days after hatching.

2. The alkaline phosphatase activity increases slowly up to 16 days and then more rapidly to 19 days, when it reaches a maximum. After the 20th day it begins to decrease until 3 days after hatching, at which time it establishes a level of activity which is apparently constant throughout the life of the chicken.

3. The acid phosphatase activity is considerably higher than the alkaline at 12 days, but shows only a slight increase up to 19 days, after which it also falls to a new level.

4. Since the rise in activity of both acid and alkaline phosphatase correspond closely to the period of cellular maturation in the chick retina, this seems to give added support to the concept that these enzymes may be associated with the histochemistry of differentiation.

<sup>5</sup> Lindeman, V. F., *Am. J. Physiol.*, 1947, **148**, 40.

considerably during early embryonic development and reaches its maximum in whole chick embryo homogenates by the 10th day of incubation. From these and previous observations (Moog<sup>2</sup>), it appears that these enzymes enter in some way into the chemistry of differentiation of tissue. Although the above findings are very convincing, nevertheless attention should be directed to the fact that the results were obtained from tissue which was not only differentiating, but also undergoing rapid growth. This is a complicating factor and does not entirely eliminate the possibility that these phosphatases may be concerned with growth rather than differentiation. It seemed to the author that this concept could be further tested by using a single tissue which could be followed through its embryonic development from the time it begins to differentiate until it becomes functional. One tissue in the chick embryo which lends itself well to such a study is the retina. According to Weysee and Burgess<sup>3</sup> differentiation of retinal cells begins about the 12th day of incubation and is complete shortly before hatching.

The present study was designed to determine whether any relationship exists between the stages of differentiation and the phosphatase activity of the embryonic chick retina.

**Material and methods.** New Hampshire Red chick embryos were used throughout this study. The eggs were obtained from a local hatchery and incubated in a commercial type incubator at a constant temperature of 102°F and a humidity of 90%. The retinas were removed from the eyes of 3 embryos of the desired age, blotted to remove excess moisture and weighed. The tissue was ground in a high speed micro grinder\* to a uniform homogenate. The homogenate was then diluted with unbuffered saline solution (Krebs Ringer solution containing approximately .03M MgSO<sub>4</sub>) so that 1 ml contained from 40-50 mg of fresh tissue. The substrate was

combined with the buffer as follows: Alkaline mixture (pH 9.1) 1.09 g disodium phenylphosphate and 10.0 g of sodium barbital per liter. Acid mixture (pH 5.0) 1.09 g disodium phenylphosphate dissolved in 800 ml of a 0.2 N sodium acetate and 200 ml 0.2 N acetic acid. The enzyme activity was measured by a method modified from Moog<sup>1</sup> as follows: All experiments were carried out in test tubes (17 × 150 mm) containing 0.4 ml of substrate mixture. After pre-heating for 5 minutes in a bath kept constant at 37.5°C, 0.1 ml of homogenate was added to each tube. The preparations were then shaken for 20 minutes and the reaction stopped by adding 4.5 ml of 10% trichloroacetic acid. The precipitate was removed by filtration and 3 ml of the clear solution was analyzed for inorganic phosphorus by the method of Fiske and SubbaRow.<sup>4</sup> Ten minutes were permitted for the blue color to develop and all measurements were made with a Coleman spectrophotometer, calibrated with a series of standard K H<sub>2</sub>PO<sub>4</sub> solutions. Experiments were always run in triplicate against 2 control tubes taken at zero time. Control values were subtracted from experimental values.

**Results and discussion.** The present study was confined to the retina from embryos beginning with the 12th day of incubation and ending approximately 3 days after hatching. This is the period during which the neural elements of the retina undergo maturation and differentiation into the various types of cells that characterize the functional organ. Although differentiation is very extensive during this time there is no appreciable change in the weight of the retina. The practice of using the weight of the tissue for computing data on chemical studies in embryonic tissues during different stages of development is sometimes considered a questionable procedure due to the changing concentration of the cytoplasm. However, since the chick retina did not increase more than 10% in weight over the period studied it seemed that there could be no serious objection in this case. The phosphatase activity is therefore expressed in terms of micrograms of inorganic

<sup>2</sup> Moog, F., *Biol. Bull.*, 1944, **86**, 51.

<sup>3</sup> Weysee, A. W., Burgess, W. S., *Am. Nat.*, 1906, **40**, 611.

\* The homogenizer is one of our own design consisting of a stainless steel drill driven by a high speed motor.

<sup>4</sup> Fiske and SubbaRow, *J. Biol. Chem.*, 1925, **66**, 375.

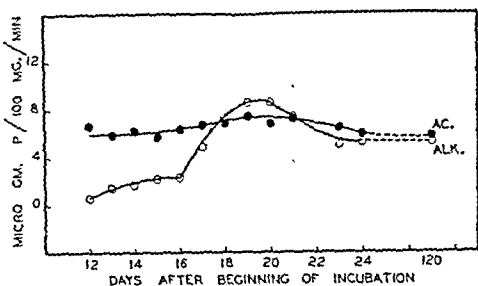


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<sup>5</sup> Lindeman, V. F., *Am. J. Physiol.*, 1947, **148**, 40.

## 17215. Infection as a Factor Causing Death in the Eviscerate Rat.

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The data of these experiments indicate that the extent of bacterial contamination during the procedure of evisceration in the rat is an important factor in determining the survival time. Survival was not affected by the administration of gelatin or of Tyrode's solution.

**Methods.** Male rats of the Sprague-Dawley strain were maintained on a diet of Archer Dog Pellets. The technic of evisceration, which is done in 2 stages, has been described by Ingle.<sup>1</sup> When the animals reached  $250 \pm 2$  g, they were anesthetized (intraperitoneal injection of 18 mg of cyclopentenyl-allyl-barbituric acid sodium) and subjected to the second stage of evisceration. Intravenous infusions of solutions containing glucose (C.P. Dextrose, Merck) and insulin (Regular Insulin, Lilly) were made by a continuous injection machine which delivered fluid into the saphenous vein of the right hind leg at the rate of 20 cc per 24 hours. The glucose load was 44 mg per 100 g of rat per hour and the insulin dose was 4 units per 24 hours per rat. Temperature was constant at  $26.5 \pm 0.5^\circ\text{C}$ .

The time of survival was determined by use of a heart-beat amplifier (Model A, Upjohn) which was designed to amplify the D.C. potential generated by the heart beat to actuate a 6-point recording mechanism (Leeds and Northrop Micromax S). This apparatus gives a permanent visual record of an all-or-none response of the amplifier to the beating heart of 6 animals simultaneously.

**Experiments and results.** It is well known that the presence of inorganic ions other than sodium and chloride favor the survival of perfused isolated organs and tissues. In Experiment 1, a comparison was made of the survival times of eviscerate rats given Tyrode's solution with the survival times of similar rats given 0.9% sodium chloride solution. The amount of added glucose and insulin was the same in each group (see methods). Twenty-one rats given Tyrode's

solution had an average survival of 2822 minutes with a range of 2457 to 3580 minutes. An equal number given saline had an average survival of 2818 minutes with a range of 2350 to 3262 minutes. It was concluded that either the use of Tyrode's solution failed to prolong survival to a greater extent than saline or that the effect was small.

As the eviscerate rat approaches death, there is a significant decrease in the concentration of proteins in the blood plasma and there is a concomitant increase in the free fluid of the body cavities (Ingle, Prestrud, and Nezamis, unpublished data). It was considered possible that the loss of fluid could be decreased and survival prolonged by the intravenous administration of a solution of colloids which might remain in the blood and sustain its osmotic activity. A 5% solution of degraded and purified gelatin containing 0.9% sodium chloride (Plazmoid, Upjohn) was used. Glucose and insulin were added to the solution. Fourteen eviscerate rats given gelatin had an average survival of 2509 minutes with a range of 1985 to 3046 minutes. Sixteen similar rats given saline with glucose and insulin had an average survival of 2748 minutes with a range of 2364 to 3076 minutes. The administration of gelatin did not reduce the rate at which fluid accumulated in the body cavities. It was concluded that this preparation of gelatin did not prolong the survival of the eviscerate rats.

Although the normal rat has a high natural resistance to the infection of wounds, the resistance of the eviscerate rat to infection is very low. A few hours following evisceration the peritoneum and even the blood becomes heavily charged with bacteria. We have never observed septicemia and peritonitis following other operations in the rat. The following organisms have been recovered from the peritoneal fluid of rats 24 hours following evisceration: *Pseudomonas*, hemolytic streptococci, *E. coli*, staphylococci, and *Proteus vulgaris*. Our attempts to apply sterile

<sup>1</sup> Ingle, D. J., *Exp. Med. and Surg.*, 1949, 7, 34.

technic, although adequate for all of the many other operative procedures which we have carried out on the rat, have been only partially successful in the eviscerate rat. The sterilization of the skin of the rat with a germicidal agent (Mercresin, Upjohn), the use of sterile instruments, the use of sterile solutions for the infusions, etc., have greatly reduced the extent of bacterial growth per unit time, and the peritoneal fluid of an occasional animal has remained sterile, but uniform success has not been attained. That infection is an important factor in limiting the survival of these animals is indicated by the following results.

In Experiment 3, 26 rats which were eviscerated without asepsis had an average survival of  $2743 \pm 109$  minutes, whereas 26 rats which were eviscerated under "aseptic" conditions had an average survival of  $3113 \pm 67$  minutes. The standard deviation of the difference (370) was 129. The ratio between the difference and its standard deviation was 2.9, thereby meeting the usual requirements of statistical significance.

*Discussion.* Although a solution containing potassium, calcium, magnesium, phosphate and bicarbonate ions in addition to sodium and chloride is superior to saline alone for the perfusion of isolated tissues, the administration of Tyrode's solution to the eviscerate rat failed to have a significantly more favorable effect on survival than physiological saline. The blood and tissues of the eviscerate rat contain an optimal concentration and distribution of these ions at the time of operation and it is probable that no lack of these substances develops during its survival.

Hepatic insufficiency is characterized by the development of ascites. The extent to which the loss of fluid from the blood into the body cavities may be due to a decrease in plasma proteins is not known to us. The administration of gelatin did not inhibit the rapid accumulation of fluid in the body cavi-

ties, nor did it prolong survival. Other plasma substitutes should be tested in the eviscerate rat.

The readiness with which the eviscerate rat develops septicemia and its low resistance to infection of the peritoneal cavity in contrast to the high resistance of the non-eviscerate rat offer interesting problems. The future success of our efforts to prolong the survival of the eviscerate rat will depend in part upon the outcome of our attempts to completely prevent infection. Even the partial success of our present efforts has significantly prolonged the survival of these animals. The average survival of the "aseptic" group of Experiment 3 is longer than has been reported in the literature for any series of liverless mammals. The longest survival of any single animal of our series to date is 63 hours and 47 minutes.

*Summary.* Eviscerate male rats were given continuous intravenous infusions of solutions containing glucose and insulin. The times of survival were determined by a heart-beat recorder. The substitution of Tyrode's solution and of 5% gelatin for 0.9% sodium chloride in the infusion fluid failed to prolong survivals. The rats which were eviscerated without sterile technic developed septicemia and peritonitis. The attempts to maintain asepsis during the procedure of evisceration were not uniformly successful, but the average survival of 26 rats operated under "aseptic" conditions was  $3113 \pm 67$  minutes as compared to  $2743 \pm 109$  minutes for 26 rats operated without asepsis. The maximum survival time recorded for any animal was 63 hours and 47 minutes.

We wish to express our appreciation to Mary Ann Nook, Department of Bacteriology, The Upjohn Laboratories, who carried out the bacteriologic studies for these experiments.

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## 17216. Experimental Lathyrism in the White Rat and Mouse.\*

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One of us (L.) has recently reported studies of the production of experimental lathyrism in the white rat.<sup>1</sup> Of the species studied, the most characteristic symptoms resulted from the ingestion of the seeds of *Lathyrus odoratus* (the flowering sweet pea) while the seeds of *Lathyrus sylvestris Wagneri* (flat pea) were most toxic, since death resulted within a few days and after the consumption of as little as 4 g of the legume. The toxic principle was readily extracted with 30% ethanol in every case.

In the present study, we report observations on the seeds of another species of *Lathyrus*, *Lathyrus latifolius* (the perennial or everlasting pea), commonly grown for its flowers in gardens.<sup>†</sup> The finely ground meal was incorporated into the diet of young male white rats (50-60 g) at the 50% level as in previous experiments.<sup>1</sup> Animals fed the *Lathyrus latifolius* meal diet showed signs of marked intoxication and died in from 3 to 8 days after the beginning of the experiment. A typical protocol in condensed form is presented here. A rat of 70.4 g gained 8 g and consumed 20 g of the diet (*i.e.*, 10 g of the legume) in the first 2 days of the experiment. On the 3rd day, 4.3 g were eaten and on the 3 following days, 0.4 to 1.0 g. Violent convulsions, marked reactions to external stimuli such as tapping the cage with a rod, and partial paralysis appeared on the 5th day and death resulted on the 6th day. A similar picture was presented in 6 other rats which received the *latifolius* meal. The symptoms were sim-

ilar in most respects to those already reported in rats fed seeds of *Lathyrus sylvestris Wagneri*. Control rats fed the edible white pea of commerce (*Pisum sativum*, var. *arvense*) ate well, gained steadily and showed no signs of toxicity. It is evident that this species of *Lathyrus* was more toxic for rats than was any of the other species previously studied in this laboratory except *L. sylvestris Wagneri*. Mr. Roland McKee of the United States Department of Agriculture in a private communication received before the experiments with *Lathyrus latifolius* were undertaken, has stated that "I have always considered this species as very closely related to *L. sylvestris*." It is of considerable interest that the toxicity of *latifolius* is so similar to that of *sylvestris Wagneri*.

Marked variations in the susceptibility of domestic or laboratory animals to *Lathyrus* intoxication have been observed.<sup>2,3</sup> We have studied in young white mice the effects of the consumption of the seeds of *Lathyrus odoratus*, the species of *Lathyrus* with which most typical symptoms of experimental lathyrism in the young white rat were observed. The diet was similar to that used in previous experiments with rats.<sup>1</sup> However, since the protein requirement of the diet of the smaller animal, the white mouse, is greater than that of the rat, the amount of casein was increased from 10 to 15%, the extra casein replacing an equivalent weight of starch. When young male white mice of 12-15 g weight were fed diets containing 50% of *odoratus* meal, they grew well until a weight of approximately 25-30 g was reached. The increments of weight were in every respect similar to those of pair-fed control mates which received diets containing *Pisum sativum*. The gains per 100

\* This work has been made possible by a grant from the Board of Governors of the Horace H. Rackham School of Graduate Studies of the University of Michigan.

<sup>1</sup> Lewis, H. B., Fajans, R. S., Esterer, M. B., Chen, C.-W., and Oliphant, M., *J. Nutrition*, 1948, **36**, 537.

<sup>†</sup> Obtained through the cooperation of Rex D. Pearce of Moorestown, N. J.

<sup>2</sup> Schuchardt, R., *Deut. Arch. Klin. Med.*, 1886-87, **40**, 312.

<sup>3</sup> Stockman, R., *J. Pharm. Exp. Therap.*, 1929, **37**, 43.

g of food consumed for the first 20 days were essentially the same, 11.8 and 11.0, respectively. Although the diet was maintained over a period of 17 weeks, no symptoms of lathyrism were observed. Since we were using a new lot of *L. odoratus* peas, we felt it necessary to confirm previous observations of the toxicity of this species for rats. Accordingly, we repeated our previous experiments<sup>1</sup> with the new shipment of sweet pea seed. All the rats developed characteristic experimental lathyrism in from 3 to 6 weeks. In view of the failure of young white mice to exhibit any symptoms of lathyrism on diets containing *odoratus*, it is notable that the mice ate from 71 to 86 g (average 77 g) of diet per 100 g of body weight over a typical 4-day period (17th to the 20th day of the experiment). This consumption was twice that of the young white rats under similar conditions (37.5 g), rats which without exception showed signs of acute lathyrism.

Since white mice fed *odoratus* showed no evidence of toxicity, it was of interest to study the effects of diets containing the 2 species of *Lathyrus*, which had proven to be most injurious to rats, i.e., *Lathyrus sylvestris* Wagneri and *Lathyrus latifolius*. Three mice received diets containing 50% *latifolius*. None showed the marked irritability, convulsions or paralysis seen in rats fed this diet, but all lost weight rapidly, decreased the food consumption and died in 5, 10 and 12 days, respectively. Two mice, fed diets containing *lati-*

*folius* extracted with 30% ethanol as previously described<sup>1</sup> maintained their weight, ate the diet well and survived with no signs of toxicity for 18 days, at which time lack of available legume resulted in the termination of the experiment.

Three mice fed diets containing 50% of *L. sylvestris* Wagneri seed lost weight rapidly and died in 4, 6, and 7 days, respectively. Increased irritability and convulsions were observed, but these symptoms were, perhaps, slightly less acute than in young white rats.

It is of some interest to note that Schuchardt<sup>2</sup> in his classical paper on lathyrism states that *Lathyrus sylvestris* and *Lathyrus latifolius* afford "essbare Samen!"

**Summary.** 1. Young white mice of 12 to 15 g body weight fed diets containing 50% *Lathyrus odoratus* meal grew well and showed no development of experimental lathyrism in 17 weeks, although young white rats which received a similar diet developed experimental lathyrism in from 3 to 6 weeks. 2. Diets containing *Lathyrus latifolius* meal (50%) were toxic for both rats and mice, death occurring in from 3 to 8 days (rats) and 5 to 12 days (mice). The extraction of seed of *Lathyrus latifolius* meal with 30% ethanol removed the toxic principle. Mice fed the extracted meal survived and appeared normal over a period of 18 days. 3. Mice fed *Lathyrus sylvestris* seed died with symptoms of acute toxicity in 4 to 7 days.

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## 17217. Glycogen in Rachitic Cartilage and Its Relation to Healing.\*

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The presence of glycogen in cartilage was described almost 100 years ago by Charles Rouget.<sup>1</sup> During the intervening years the feeling has grown that glycogen might have some relation to the calcification process.

Marchand<sup>2</sup> noted the high glycogen content of hypertrophic cartilage cells in the region of lime salt deposition. Hoffmann *et al.*<sup>3</sup> observed that "a striking relationship exists between the disappearance of glycogen in car-

\* Aided by a grant from Mead Johnson and Company.

<sup>1</sup> Rouget, C., *J. de la Physiol.*, 1859, 2, 308.

<sup>2</sup> Marchand, F., *Firch. Arch.*, 1885, 100, 42.

<sup>3</sup> Hoffmann, A., Lehmann, G., and Wertheimer, E., *Pflüger's Arch.*, 1928, 220, 183.

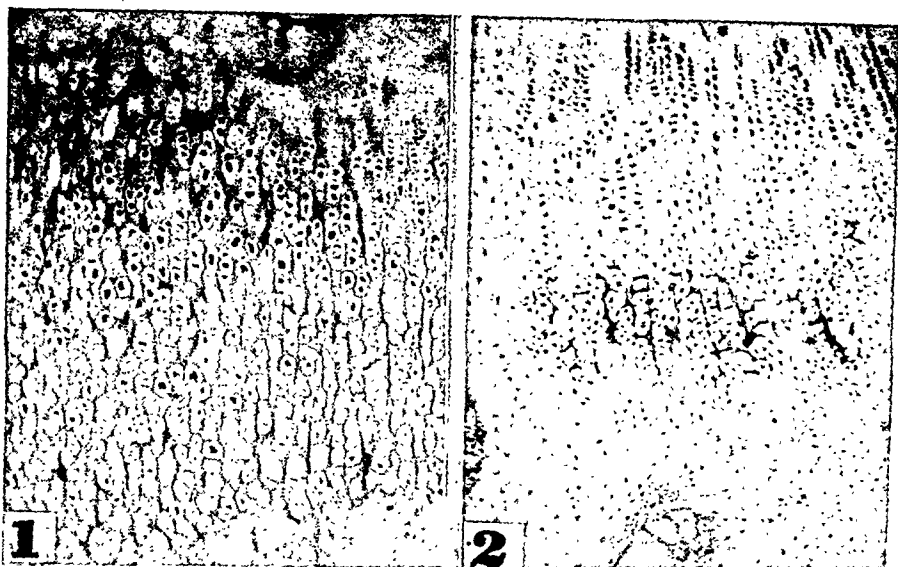


FIG. 1.

Epiphyseal cartilage of rachitic rat. Note widened zone of hypertrophic cells, the uppermost ones of which contain dark accumulations of glycogen. The cell nuclei stain poorly and should not be confused with the glycogen. Periodic acid-leucofuchsin technic.

FIG. 2.

Epiphyseal cartilage of rachitic rat (same magnification as Fig. 1) which had been given phosphate intraperitoneally 24 hours before. Note initial deposition of inorganic material in upper portion of hypertrophic cartilage corresponding to zone in which glycogen is found. Von Kossa technic.

tilage and its ossification." Harris,<sup>4</sup> shortly thereafter, suggested that glycogen might have a bearing on the process of calcification, possibly being related to Robison's<sup>5</sup> phosphatase mechanism. More recently, the presence of glycogen in hypertrophic cartilage cells has assumed further importance as a result of Gutman's<sup>6</sup> demonstration of phosphorylase activity in epiphyseal cartilage.

The following observations, which are part of a general histochemical survey of normal and diseased cartilage and bone,<sup>7</sup> are briefly reported because of the further evidence they bring to bear on the relationship of glycogen to the calcification mechanism. Weanling rats were placed on the Steenbock rachitogenic diet (No. 2965) supplemented with

crystalline vitamins. After one to 5 weeks on this regimen some of the rats were injected intraperitoneally with 2.5 cc per 100 g of animal of a mixture of 1 part M/10  $\text{NaH}_2\text{PO}_4$  and 4 parts M/10  $\text{Na}_2\text{HPO}_4$  to produce healing as recommended by Urist and McLean.<sup>8</sup> Such animals were killed from 12 to 24 hours afterward; uninjected rachitic controls were likewise sacrificed. The upper end of the tibia was fixed in neutral formal-alcohol and, after sectioning without decalcification, stained for glycogen by the Bauer or McManus technics and for inorganic materials by the von Kossa procedure.

Glycogen is not present in the widened zone of hypertrophic cells, so characteristic of rickets. Glycogen is present in the usual increasing amounts in the cells which are undergoing the normal maturation cycle at the upper margin of the widened zone of hypertrophic cells. In other words, glycogen

<sup>4</sup> Harris, H. A., *Nature*, 1932, **130**, 996.

<sup>5</sup> Robison, R., *Biochem. J.*, 1923, **17**, 286.

<sup>6</sup> Gutman, A. B., and Gutman, E. B., *Proc. Soc. Exp. Biol. and Med.*, 1941, **48**, 687.

<sup>7</sup> Follis, R. H., Jr., and Berthrong, M., *Am. J. Path.*, 1948, **24**, 685.

<sup>8</sup> Urist, M. R., and McLean, F. C., *J. Bone and Joint Surg.*, 1941, **23**, 283.

appears in normal fashion as the cartilage cell reaches its full stage of differentiation; as the cell persists, however, for reasons which are not clear at this time, the glycogen disappears. In the rachitic animals in which healing had not been induced, no deposition of inorganic material is present in the vicinity of the hypertrophic glycogen containing cells, or those from which glycogen has disappeared. When healing was instituted, it took place, as has often been noted both *in vivo*<sup>9</sup> and *in vitro*,<sup>10</sup> in the matrix adjacent to those cartilage cells which have most recently matured, that is, in the immediate vicinity of the cells which contain abundant glycogen and, in the early period of observation, not down farther in the matrix adjacent to the hypertrophic cells devoid of glycogen. There is thus a regional relationship between the presence of glycogen and deposition of inorganic elements as demonstrated by the von Kossa technic. It is, of course, impossible to determine any precise reciprocal relationship between the disappearance of glycogen and the appearance of inorganic materials since to follow the disappearance of one and the appearance of the other is not possible. It is hoped that quantitative methods<sup>11</sup> now available, using larger animals, can be applied to study this particular problem.

The glycogen content of rachitic cartilage has received little attention. Some years ago Suppes<sup>12</sup> reported observations on postmortem human material and concluded that rachitic cartilage did not contain glycogen. No mention was made of the presence of glycogen in the uppermost hypertrophic cells so that one suspects autolytic changes may have been a complicating factor.

The localization of glycogen in rachitic cartilage in the region where calcification first appears during healing is, of course, important in any theory of calcification which has the glycogenolytic cycle as its basis.<sup>13</sup> More precise localization of phosphorylase activity<sup>6</sup> as well as that of other enzymes which participate in the cycle will have to be carried out before such an hypothesis can be fully accepted.

**Summary.** Glycogen is present in rachitic cartilage only in the most recently matured hypertrophic cells. The broad zone of hypertrophic cells beneath is devoid of glycogen. This localization coincides with the area where inorganic elements deposit during the initial stages of healing and furnishes further evidence for the relationship of glycogen, and possibly the glycogenolytic cycle, to the calcification mechanism.

<sup>12</sup> Suppes, J., *Frank. Z. Path.*, 1922, 26, 268.

<sup>13</sup> Gutman, A. B., *Trans. Conf. on Metabolic Aspects of Convalescence*, Josiah Macy, Jr. Foundation, 1946, 14, 20.

Received May 9, 1949. P.S.E.B.M., 1949, 71.

<sup>9</sup> McCollum, E. V., Simmonds, N., Shipley, P. G., and Park, E. A., *J. Biol. Chem.*, 1922, 51, 41.

<sup>10</sup> Shipley, P. G., Kramer, B., and Howland, J., *Biochem. J.*, 1926, 20, 379.

<sup>11</sup> Follis, R. H., Jr., *Fed. Proc.*, 1949, 8, 480.

## 17218. Simultaneous Administration of Adrenal Cortical Extract and Desoxycorticosterone; Effects on Blood Pressure of Hypertensive Patients.\*

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*From the Department of Medicine, Columbia University College of Physicians and Surgeons, and the Presbyterian Hospital in New York City.*

Previous studies<sup>1</sup> have indicated that the

continued administration of an adrenal cortical extract may be associated with a decrease in the "resting" blood pressure of patients with

\* This investigation was supported, in part, by a research grant from the National Heart Institute, U. S. Public Health Service, and was aided through the generosity of the Albert and Mary Lasker Foundation.

<sup>1</sup> Pines, K. L., Perera, G. A., Vislocky, K., and Barrows, A. D., *Proc. Soc. Exp. Biol. and Med.*, 1948, 68, 286.

TABLE I.  
Laboratory Data of 5 Hypertensive Patients Before and During Treatment with DCA and ACE.

Wt, k	Urine*		Serum											
	Vol. cc	Sodium milliequiv.	Hematocrit % cells	Urea N, mg/100 cc		Proteins, g/100 cc		Na		Cl milliequivalents/l		K		
A†	P‡		A	P	A	P	A	P	A	P	A	P	A	P
65.5-66.0	1840-1580	96-64	40.3-37.3	17-15	6.2-6.1	140.0-142.3	—	99.6-102.0	—	3.8-3.3				
65.6-66.3	1400-1400	57-42	46.0-46.0	10-8	6.4-6.4	139.3-138.3	—	102.2-100.2	—	4.7-4.2				
78.5-78.4	1460-1520	74-56	—	—	—	136.3-137.4	—	—	—	4.4-4.3				
63.2-64.0	1540-1400	84-60	45.0-44.1	12-10	6.6-6.5	138.9-138.6	—	99.2-100.0	—	3.9-3.1				
65.0-65.6	1620-1480	—	—	—	7.5-7.4	138.6-138.7	—	—	—	5.2-5.3				

\* Mean daily values for week preceding and week of steroid administration.

† A = Start of treatment.

‡ P = End of treatment.

uncomplicated hypertensive vascular disease. Inasmuch as the daily injection of desoxycorticosterone acetate has consistently produced at least a transitory and sometimes a sustained pressor response under comparable conditions,<sup>2,3</sup> the effect of both agents was observed when given in combination.

**Methods.** Two men and 3 women with uncomplicated hypertensive vascular disease were studied on the metabolism ward of the Presbyterian Hospital. The criteria in selection of patients and the methods employed were identical to those previously described.<sup>4</sup> All subjects were maintained on a constant dietary, fluid and sodium chloride intake. Following a 3-week baseline period, desoxycorticosterone acetate† (DCA) was administered subcutaneously in 5 mg doses twice daily, and adrenal cortical extract (Upjohn) (ACE) was given intramuscularly in 10 cc doses twice daily, both materials being injected for

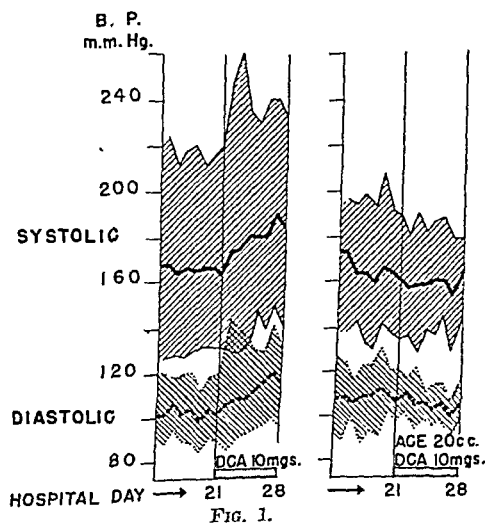


FIG. 1.

Maximum, minimum and mean systolic and diastolic blood pressures of 12 hypertensive patients given DCA and 5 given both DCA and ACE.

<sup>2</sup> Perera, G. A., and Blood, D. W., *Ann. Int. Med.*, 1947, **27**, 401.

<sup>3</sup> Perera, G. A., *Proc. Soc. Exp. Biol. and Med.* 1948, **68**, 48.

<sup>4</sup> Perera, G. A., and Blood, D. W., *J. Clin. Invest.*, 1947, **26**, 1109.

† Desoxycorticosterone acetate (DOCA) was supplied through the generosity of Dr. K. W. Thompson of Roche-Organon, Inc., Nutley, N. J.

a period of a week. The results were compared to those previously obtained in 14 patients receiving only DCA,<sup>2</sup> two of whom being included in the present series after repeatedly demonstrating a prompt rise in blood pressure in association with this steroid given alone.

**Results.** The combination of DCA and ACE generally produced increases in weight, hemodilution, reductions in urinary volume, and sodium and chloride retention. These changes were similar to those observed after DCA alone. No consistent alterations in serum sodium or chloride values were noted.

but reductions in serum potassium levels were again evident in the majority (Table I). In no instance was fever, leucocytosis, eosinophilia or rise in erythrocyte sedimentation rate produced by these agents. In all patients there were either minimal changes in "resting" blood pressure or an actual decline (Fig. 1).

**Conclusions.** In patients with hypertensive vascular disease, the simultaneous administration of an adrenal cortical extract appears to block the pressor effect of desoxycorticosterone acetate when used alone.

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### 17219. Effect of 11-Desoxycorticosterone Acetate upon Carbohydrate Utilization by the Depancreatized Rat.\*

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*From The Scripps Metabolic Clinic, La Jolla, Calif.*

In recent years it generally has been thought that only those steroids of the adrenal cortex which have an oxygen atom at the 11 position in ring C can directly influence carbohydrate metabolism. In particular, the synthetic compound 11-desoxycorticosterone, which has not even been demonstrated with certainty to occur in the gland and which is exceedingly potent in causing sodium retention, has been considered to have no carbohydrate action. However, Verzar and his co-workers have demonstrated<sup>1</sup> that this steroid may reduce glycogen synthesis in isolated diaphragm muscle and may prevent the increased glycogen formation produced in this tissue by insulin. Using glucose labeled with carbon 14, this observation has been confirmed and extended in this laboratory.<sup>2</sup>

Ingle<sup>3</sup> recently has found that in rats made

diabetic by partial pancreatectomy, 11-desoxycorticosterone acetate caused a slight decrease in the level of urinary glucose when given in small doses, while large doses of the steroid led to a marked exacerbation of the glycosuria.

Our experiments were carried out in an attempt to find out if desoxycorticosterone would oppose the action of insulin in the diabetic organism.

**Methods.** Male albino rats of the Slonaker strain<sup>4</sup> were depancreatized at 6 weeks of age in the usual manner.<sup>5</sup> The rats were maintained on Purina Chow until 1 week before the start of the experiment at which time they were fed the following rather high carbohydrate diet: Sucrose 50, casein 18, yeast 10, salt mixture 4, C.L.O. 4, lard 14. After their diabetes was stabilized on this above diet, the animals were placed in individual metabolism cages. The rats were weighed and their food intake determined daily. The urine was

\* This investigation was supported by a research grant from the Division of Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service.

<sup>1</sup> Verzar, F., and Wenner, V., *Biochem. J.*, 1948, **42**, 35; 1948, **42**, 48.

<sup>2</sup> Bartlett, G. R., Wick, A. N., and MacKay, E. M., *J. Biol. Chem.*, 1949, **178**, 1003.

<sup>3</sup> Ingle, Dwight J., *Proc. Soc. Exp. Biol. and Med.*, 1948, **69**, 329.

<sup>4</sup> MacKay, L. L., *Am. J. Physiol.*, 1928, **86**, 215.

<sup>5</sup> Pauls, Frances, and Drury, Douglas R., *J. Biol. Chem.*, 1942, **145**, 481.

TABLE I.  
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Wt, k	At P†	Urine*		Serum									
		Vol, cc	Sodium milliequiv.	Chloride milliequiv.	Hematocrit % cells	Urea N, mg/100 cc	Proteins, g/100 cc	Na	Cl	K			
					A P	A P	A P	A P	A P	A P	A P	A P	A P
65.5-66.0		1840-1580	90-64	85-50	40.3-37.3	17-15	6.2-6.1	140.0-142.3	99.6-102.0	3.8-3.3			
65.6-66.3		1400-1400	57-42	69-58	46.0-46.0	10- 8	6.4-6.4	139.3-138.3	102.2-100.2	4.7-4.2			
78.5-78.4		1460-1520	74-56	94-75	—	—	—	136.3-137.4	—	4.4-4.3			
63.2-64.0		1540-1400	84-60	98-72	45.0-44.1	12-10	6.6-6.5	138.9-138.6	99.2-100.0	3.9-3.1			
65.0-65.6		1620-1480	—	118-100	—	—	7.5-7.4	138.6-138.7	—	5.2-5.2			

\* Mean daily values for week preceding and week of steroid administration.

† A = Start of treatment.

P = End of treatment.

uncomplicated hypertensive vascular disease. Inasmuch as the daily injection of desoxycorticosterone acetate has consistently produced at least a transitory and sometimes a sustained pressor response under comparable conditions,<sup>2,3</sup> the effect of both agents was observed when given in combination.

**Methods.** Two men and 3 women with uncomplicated hypertensive vascular disease were studied on the metabolism ward of the Presbyterian Hospital. The criteria in selection of patients and the methods employed were identical to those previously described.<sup>4</sup> All subjects were maintained on a constant dietary, fluid and sodium chloride intake. Following a 3-week baseline period, desoxycorticosterone acetate<sup>†</sup> (DCA) was administered subcutaneously in 5 mg doses twice daily; and adrenal cortical extract (Upjohn) (ACE) was given intramuscularly in 10 cc doses twice daily, both materials being injected for

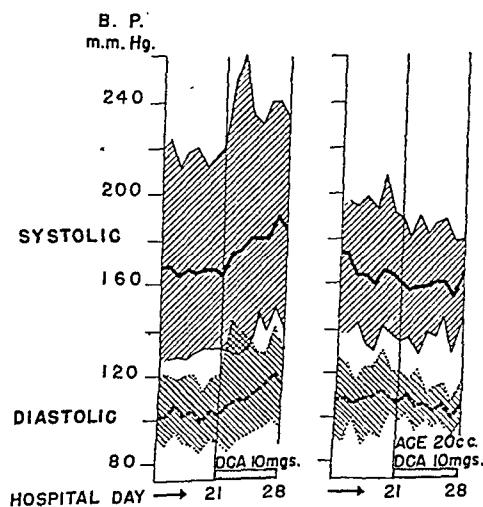


Fig. 1.

Maximum, minimum and mean systolic and diastolic blood pressures of 12 hypertensive patients given DCA and 5 given both DCA and ACE.

<sup>2</sup> Perera, G. A., and Blood, D. W., *Ann. Int. Med.*, 1947, **27**, 401.

<sup>3</sup> Perera, G. A., *Proc. Soc. Exp. Biol. and Med.* 1948, **68**, 48.

<sup>4</sup> Perera, G. A., and Blood, D. W., *J. Clin. Invest.*, 1947, **26**, 1109.

<sup>†</sup> Desoxycorticosterone acetate (DOCA) was supplied through the generosity of Dr. K. W. Thompson of Roche-Organon, Inc., Nutley, N. J.

## 17220. Effect of Folic Acid, "4-Amino" Folic Acids and Related Substances on Growth of Chick Embryo.\*

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(Introduced by C. P. Rhoads.)

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Pteroylglutamic acid (folic acid) has an essential role in the growth of bacteria, protozoa, insects, birds and mammals.<sup>1</sup> Several weak folic acid antagonists were first described in 1947,<sup>2,3</sup> and shortly afterwards Seeger *et al.*<sup>4</sup> discovered 4-amino pteroylglutamic acid, an extremely powerful antagonist to folic acid by the *Streptococcus fecalis* R test. Franklin *et al.*<sup>5</sup> found that 4-amino PGA was toxic to mice, and that folic acid offered only slight protection, and this within a narrow dose range. 4-amino PGA and some of its related compounds produced a toxic picture in mammals somewhat similar to that of other systemic cell poisons, such as x-rays and nitrogen mustard, with lymph node regression, leucopenia and thrombocytopenia with bone marrow aplasia, diarrhea with sloughing of the intestinal mucosa, and death within 3 to 4 days after a single acute dose. Philips, Thiersch *et al.*<sup>6,7,8</sup> have reviewed the

pharmacology of these compounds and they conclude that the "4-amino" compounds are true folic acid antagonists, but are unique as anti-metabolites in that they induce an acute and absolute folic acid deficiency physiologically, even in the presence of folic acid. Because of the actions of the "4-amino" folic acids, a study was made of the effect of these and related compounds on the growth of the chick embryo.

**Methods.** Fertile White Leghorn eggs were obtained from a commercial source. The eggs were incubated at 100°F. and a relative humidity of 75%. In most instances the embryos were injected with the test agent at 4 days of incubation. The chemicals were dissolved in sterile saline and injected into the yolk sac by introducing a No. 25 needle through a small hole at the blunt end of the egg. The total volume injected into each egg ranged between 0.1 and 0.2 cc; larger volumes were sometimes necessary for the large doses of the relatively non-toxic drugs. When most of the embryos in a given group were dead, those remaining were sometimes sacrificed for detailed examination. The toxicity experiments were usually concluded by sacrificing the embryos 8 to 10 days after injection, when the embryos were 12 to 14 days of age.

The 15 compounds used in this study are listed in Table I.

**Results.** After considerable exploration, the 4 day old embryo was selected as most

\* This study was aided by a grant from the Jane Coffin Childs Memorial Fund for Medical Research and the National Cancer Institute of the National Institute of Health, United States Public Health Service. Most of the drugs used were supplied by the Lederle Laboratories Division and the Calco Laboratories of the American Cyanamid Company; 2,6-diaminopurine was supplied by the Wellcome Research Laboratories.

<sup>1</sup> Jukes, T. H., and Stokstad, E. L. R., *Physiol. Rev.*, 1948, **28**, 51.

<sup>2</sup> Franklin, A. L., Stokstad, E. L. R., and Jukes, T. H., *Proc. Soc. Exp. Biol. and Med.*, 1947, **65**, 368.

<sup>3</sup> Hutchings, B. L., Mowat, J. H., Oleson, J. J., Stokstad, E. L. R., Boothe, J. H., Waller, C. W., Angier, R. B., Semb, J., and Subbarow, Y., *J. Biol. Chem.*, 1947, **170**, 323.

<sup>4</sup> Seeger, O. R., Smith, J. M., Jr., and Hultquist, M. E., *J. Am. Chem. Soc.*, 1947, **69**, 2567.

<sup>5</sup> Franklin, A. L., Stokstad, E. L. R., and Jukes, T. H., *Proc. Soc. Exp. Biol. and Med.*, 1948, **67**, 398.

<sup>6</sup> Philips, F. S., and Thiersch, J. B., *J. Pharm. and Exp. Therap.*, 1949, **95**, 303.

<sup>7</sup> Philips, F. S., Thiersch, J. B., and Ferguson, F. C., *Ann. New York Acad. Sci.*, in press.

<sup>8</sup> Thiersch, J. B., and Philips, F. S., *Am. J. Med. Sci.*, 1949, **217**, 575.

<sup>9</sup> Hitchings, G. H., Elion, G. B., VanderWerff, H., and Fako, E. A., *J. Biol. Chem.*, 1948, **174**, 765.



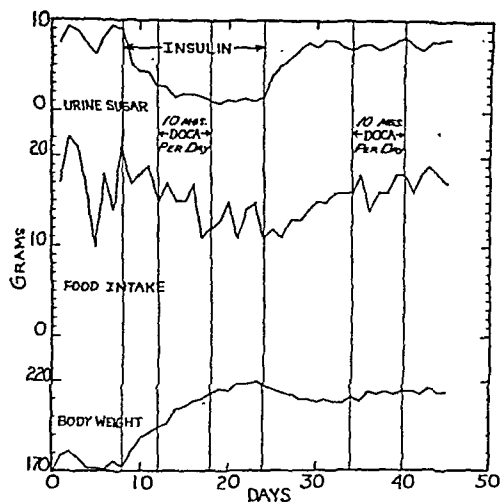


FIG. 1.

Effect of insulin and DOCA on food intake, urine sugar and body weight in depancreatized rats.

collected with a preservative in 24 hour samples and sugar excretion determined by the Shaffer-Somogyi method.<sup>6</sup> Protamine zinc insulin<sup>†</sup> was administered in doses of 1 unit 2 times daily. Desoxycorticosterone acetate (DOCA)<sup>‡</sup> was made up in oil at a concentration of 10 mg per ml. It was given by subcutaneous injection in divided doses each morning and late afternoon. Food was given *ad libitum*.

**Results.** Fig. 1 presents the significant data as averages for 6 rats. The figures for the individual rats show no important differences

<sup>6</sup> Shaffer, P. A., and Somogyi, M., *J. Biol. Chem.*, 1933, **100**, 695.

<sup>†</sup> We are indebted to Dr. F. B. Peek of the Lilly Research Laboratories for a supply of insulin.

<sup>‡</sup> Dr. Edward Henderson of the Schering Corporation supplied the desoxycorticosterone acetate ("Cortate") in oil which was used.

from the averages. As usual insulin decreased the food intake and increased the weight gain by reducing the glycosuria. The desoxycorticosterone acetate in a dose of 10 mg per rat per day for 6 days had no influence upon the action of insulin. The DOCA also had no influence when given in the same dose without insulin during an intense diabetes.

**Discussion.** At first glance our results appear to be at variance with those reported by Ingle.<sup>3</sup> This investigator's rats had a milder diabetes than ours and were force fed with a constant food intake. In our experiments the food intake varied with the desire of the animals. Had the food intake been fixed at the high pre-insulin level during the period of insulin administration, the DOCA administration might have opposed the insulin action, which was largely one of storing sugar.<sup>5</sup> It does seem odd that when no insulin was being given, DOCA in large doses failed to affect the glycosuria. This difference from Ingle's results may be due to the greater severity of the diabetes in our rats which may have been so severe that an exacerbation was not possible.

**Summary.** Severely diabetic depancreatized male rats reacted as usual to protamine zinc insulin administration by reducing their food intake but gaining weight through a reduction in the glycosuria. Desoxycorticosterone acetate in large doses was without measurable influence on the body weight gain or glycosuria. The glycosuria of the severely diabetic rat without insulin therapy and receiving food *ad libitum* was unaffected by similar doses of desoxycorticosterone acetate.

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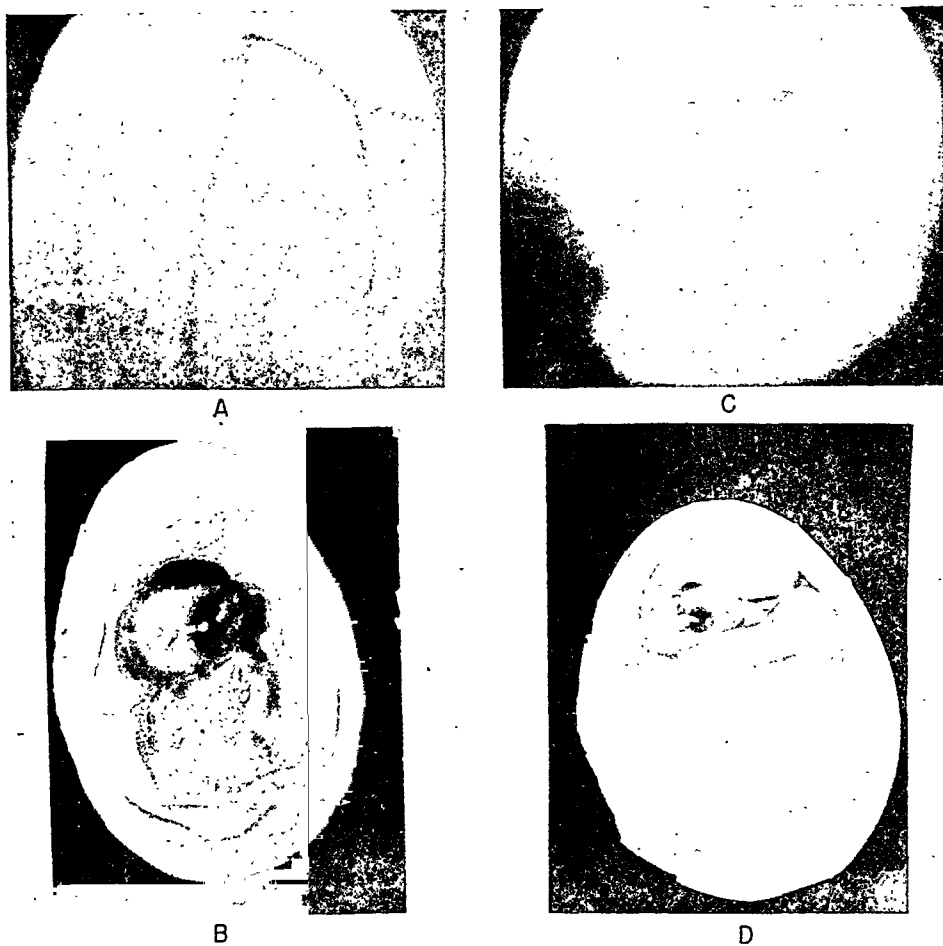


FIG. 1.

Appearance of a normal and stunted embryo, on candling, and in the opened egg. The normal appearing embryo received 0.003 mg/egg of 4-amino 9-methyl PGA at 4 days of age, sacrificed at 11 days of age. A) The vascular pattern, as seen by candling, appears normal. B) The embryo appears to be normally developed, and the allantois has extended laterally to envelop the contents of the egg. The abnormal embryo received 0.004 mg/egg of 4-amino 9-methyl PGA at 4 days of age, sacrificed at 11 days of age. C) The vascular pattern of the allantois, as seen by candling, is limited to a small area, about  $3 \times 3$  cm, and it is separated from the yolk vessels. D) The embryo is severely stunted and abnormal, the amniotic sac is relatively enlarged, and the allantois is a large sac full of clear liquid.

satisfactory for routine use. Viability was easily determined by candling. The 4 day old embryos seemed to survive toxic doses of the "4-amino" folic acids for longer periods than the younger embryos, and developmental abnormalities were more conspicuous in those embryos surviving for longer periods. Older embryos required larger doses of the drugs, and developmental abnormalities were not as apparent.

Toxicity determinations in the chick embryo are subject to considerable variability, which may be attributed to some extent to differences in the genetic and nutritional background of the embryo, the season of the year, the age of the embryo at treatment, and the trauma associated with the injection. The  $LD_{50}$  figures are, therefore, approximate and represent the minimum dose range in which the compound shows activity. The

TABLE I. Toxicity of Folic Acid and Allied Compounds to the 3 Day Old Chick Embryo.

Compound	Dose mg	No. embryos	Embryo deaths, or sacrifices ( ) or†										Approx. LD 50, mg/egg	Abnormality incidence in embryos surviving beyond 9 days of age
			5	6	7	8	9	10	11	12	13	14		
I. Pteroyl glutamic acid	20	15	0	0	2	1	0	0	0	0	(9)	(3)	20	0/12
II. " diglutamic acid	20	5	0	0	0	0	1	0	0	0	(4)	—	20	0/4
III. " triglutamic "	20	8	0	0	0	1	0	1*	0	0	(6)	—	20	1/7*
IV. 4-amino pteroylglutamic acid	.008-.010	29	0	14	10	2	(1)	11	—	—	—	—	—	2/2
	.004-.006	37	0	5	7	2	5†	4	5†	13	0	(3)	.003	8/17
V. 4-amino N <sup>10</sup> methyl pteroylglutamic acid	.002-.003	16	0	0	0	0	2	4	2	1	15	(1)	—	7/14
	.009-.013	16	1	7	0	1	2	(2)	0	2	1	—	—	5/5
	.005-.006	16	2	3	1	0	2	1	0	(1)	(3)	—	.005	1/8
VI. 4-amino 9 methyl pteroylglutamic acid	.010	6	0	0	0	0	0	0	0	0	(6)	—	—	1/6
	.004-.005	11	0	4	4	1	0	0	0	0	(2)	—	—	0/2
VII. 4-amino 9-10 dimethyl pteroylglutamic acid	.001-.003	17	1	3	3	3	2	2	1	1	(1)	—	.003	2/5
	.004-.005	21	2	2	2	2	0	1	1	1	15	(1)	—	2/13
VIII. 4-amino pteroylaspartic acid	.002-.003	23	0	4	9	2	4	11	0	(2)	—	—	—	3/4
	.400-.600	20	1	1	1	1	2	2	2	17	1	(1)	.003	4/14
	.150-.250	15	2	9	0	2	2	—	—	—	—	—	—	1/1
	.050-.100	15	0	6	4	3	1	0	(1)	—	—	—	.075	7/9
IX. 4-amino pteroylthreonine	.025	9	0	0	0	0	0	0	0	0	(5)	(4)	—	0/9
	.45	8	0	1	2	4	0	1	—	—	0	(9)	—	1/1
X. 4-amino pteroylasparagine	2	9	0	2	0	2	0	0	1	22	—	—	1.5	5/5
	0.5-1	10	2	0	0	0	0	0	1	1	2	(5)	—	1/8
	2	5	1	0	2	2	—	—	—	—	—	—	—	1/3
XI. N <sup>10</sup> methyl pteroleic acid	1	5	0	1	1	0	0	0	0	0	0	(3)	1.5	0/3
	0.5	5	0	0	1	0	0	0	0	0	0	(3)	—	0/4
	5-6	21	6	8	1	2	0	0	(1)	0	(3)	—	—	0/3
XII. Pteroylaspartic acid (dextro)	4	5	1	1	0	0	0	0	0	0	0	(3)	4	0/10
	2	15	2	2	0	0	1	0	(2)	(4)	—	—	—	0/5
XIII. Pteroylaspartic acid (racemic)	20	6	1	0	0	0	0	0	0	0	(4)	—	20	0/6
	10	5	0	0	0	0	0	0	0	0	(6)	—	—	0/4
XIV. 9-methyl pteroylglutamic acid	20	6	0	0	0	1	0	0	0	0	(4)	—	20	0/5
	10	17	0	0	0	0	0	0	0	0	(3)	(2)	10	0/7
	4-6	43	8	11	0	4	2	1	0	(1)	(16)	—	—	0/18
XV. 2,6-diaminopurine	2	12	0	5	1	0	1	0	(1)	0	—	—	—	0/10
	4-5	41	6	5†	3	8	6†	—	—	—	—	—	1	See description below (in text)
	2-3	27	2	1	2	1	1	0	22	25	2	4†	—	
	1-1.5	15	1	0	0	1	6†	1	0	2	1	(2)	—	
	.25-.50	6	0	0	0	0	1	0	0	2	—	—	—	

\* A stunted, anophthalmic embryo, not typical of "4-amino" PGA; this probably represents a spontaneous abnormality.

days old; it is unlikely that many can survive beyond this age.

These embryo abnormalities were not produced by pteroyl aspartic acid (dextro) (XII) and (racemic) (XIII), by 9 methyl pteroylglutamic acid (XIV), also weak folic acid antagonists<sup>2,3</sup> and by N<sup>10</sup> methylpterioic acid (XI).

2,6-diaminopurine is included in this report because Hitchings *et al.*<sup>9</sup> found that it acted as an antagonist of folic acid over a limited range, and as an antagonist of adenine over a more extended range, in the growth of *Lactobacillus casei*. It also damages the hematopoietic system of laboratory animals,<sup>10</sup> and prolongs the survival time of mice with transplanted leukemia,<sup>11</sup> actions qualitatively similar to those of the "4-amino" antifolics.<sup>6,12</sup>

In the 4-day-old chick embryo, 2,6-diaminopurine is a moderately toxic compound, the LD<sub>50</sub> being in the range of 1 mg/egg. The mortality pattern seems to differ appreciably from the "4-amino" compounds in that, in the LD<sub>50</sub> range, many of the embryos may survive for longer than 5 days. At this dose, the embryos surviving for longer than 5 days did not appear appreciably stunted, but they were extraordinarily pale and a few random hemoglobin determinations showed values much lower than the normal. Probably as a result of the anemia, cardiac enlargement was also conspicuous in some of these embryos. Other findings were less constant, and they included generalized edema, abnormal lower beaks, and necrotic areas in the liver. Daniel *et al.*<sup>13</sup> found that 2-amino-4-hydroxy-6,7-dimethyl pteridine and 2-amino-4-hydroxy-6,7-diphenylpteridine inhibited growth and hemoglobin formation in the chick, but the effect could be counteracted by folic acid.

The toxicity of 2,6-diaminopurine in the egg, however, could not be altered by adenine (20 mg/egg) or by folic acid (10 mg/egg).

Attempts to determine whether folic acid provided the chick embryo with some protection against the "4-amino" compounds have been unsuccessful. Twenty mg of folic acid were given almost simultaneously with different dose levels (multiples of the approximate LD<sub>50</sub>) of 4-amino PGA (IV) and 4-amino N<sup>10</sup> methyl PGA (V), a weight ratio of 1000 to 4000 to 1, but no clear-cut protection could be demonstrated. There were several experiments in which folic acid protected the chick embryo against 4-amino pteroyl aspartic acid, but this could not be demonstrated consistently. There is no evidence in these experiments that folic acid, within the closed system provided by the egg, gave any significant protection against the "anti-folic" compounds; in fact, the stunted embryos, produced by the "4-amino" antifolics, occurred in the presence of folic acid.

*Discussion.* It is not clear whether the profound stunting of the chick embryo produced by the "4-amino" anti-folics is due to a general interference with the growth of the embryo or due to injury to a specific tissue which leads to retardation in the growth of the embryo. Wagley and Morgan<sup>14</sup> found that the "4-amino" PGA's in the chick embryo produced marked cytological changes in the yolk sac blood islets, and we have observed that the vascular network of the chorioallantoic membrane is considerably reduced in size in the stunted embryos. Hertz and Tullner,<sup>15</sup> in demonstrating that "4-amino" PGA inhibits the response of the chick oviduct to estrogen stimulation, also implied that it acted on specific tissues. We tend to favor the more general interpretation, however, that the "4-amino" folic acids interfere with the growth of tissues—those growing more rapidly being most obviously affected—and the thesis that it interferes with the growth of certain tissues, in a highly specific fashion, remains to be proved.

<sup>10</sup> Phillips, F. S., and Thierch, J. B., *Fed. Am. Soc. Exp. Biol., Proc.*, 1949, **8**, 325.

<sup>11</sup> Burchenal, J. H., Bendich, A., Brown, G. B., Elion, G. B., Hitchings, G. H., Rhoads, C. P., and Stock, C. C., *Cancer*, 1949, **2**, 119.

<sup>12</sup> Burchenal, J. H., Burchenal, J. R., Kushida, M., Johnston, S., and Williams, B. S., *Cancer*, 1949, **2**, 113.

<sup>13</sup> Daniel, L. J., Scott, M. L., Norris, L. C., and Heuser, G. F., *J. Biol. Chem.*, 1949, **173**, 123.

<sup>14</sup> Wagley, P. F., and Morgan, H. R., *Bull. Johns Hopkins Hosp.*, 1948, **83**, 275.

<sup>15</sup> Hertz, R., and Tullner, W. W., *Endocrinology*, 1949, **44**, 278.

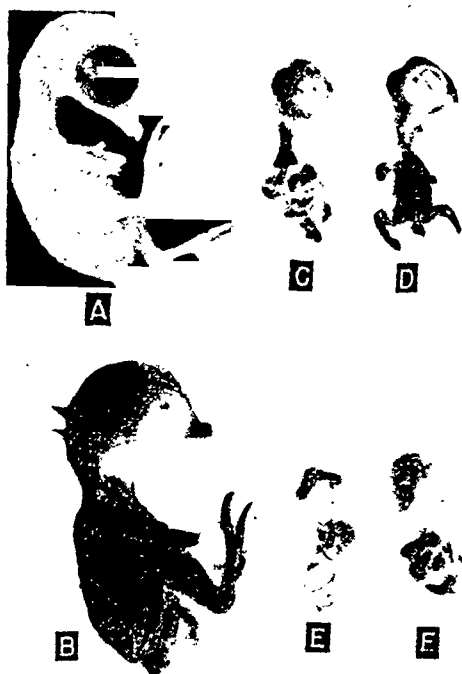


FIG. 2.

Appearance of normal and "4-amino" folic acid treated embryos. These were treated at 4 days of age and sacrificed 8 to 9 days later.

A. 12 day, control.

B. 13 day, control.

C. 2 mg 4-amino pteroylthreonine, sacrificed at 12 days.

D. 0.005 mg 4-amino 9-10 dimethyl PGA, sacrificed at 12 days.

E. 0.010 mg 4-amino N<sup>10</sup> methyl PGA, sacrificed at 13 days.

F. 0.100 mg 4-amino pteroylaspartic acid, sacrificed at 13 days.

toxicity data for the compounds tested are summarized in Table I

Folic acid and its conjugates, pteroyl mono-(I), di-(II) and tri-(III) glutamic acids do not have an appreciable toxicity in the chick embryo at doses up to 20 mg/egg.

The "4-amino" folic acids produce a uniform toxic effect on the chick embryo, but alterations in the "4-amino" anti-folic molecule may affect the LD<sub>50</sub> dosage. The LD<sub>50</sub> of 4-amino PGA (IV) is 0.003 mg/egg; this LD<sub>50</sub> dose is not appreciably altered by placing a methyl group in the N<sup>10</sup> position (V) and in the 9 position (VI), and in the 9 and 10 position (VII). Substituting aspartic acid (VIII), threonine (IX) and alanine (X)

for glutamic acid, however, increases the LD<sub>50</sub> to approximately 0.075, 1.5 and 1.5 mg/egg, respectively.

The abnormalities produced in the embryo by the "4-amino" compounds have been remarkable and fairly uniform. The embryos dying during the first 4 days after treatment are severely stunted, but they are usually too autolyzed to permit a useful examination. Those embryos surviving for 8 to 10 days show the most remarkable changes. The appearances of the allantoic vessels in an 11 day normal and abnormal embryo are shown in Fig. 1, A,C. On opening these eggs the reason for the vascular abnormality is apparent. (Fig. 1, B, D). The normal embryo weighed about 4 g and the chorioallantoic membrane extended widely. The treated embryo was severely stunted, weighing about 0.8 g. The amnion was distended with clear fluid. The allantois remained as a large fluid-filled sac, which was in connection with the shell over a relatively small area, and its blood vessel network was appropriately restricted.

Several abnormal embryos and controls of the same age are shown in Fig. 2. These embryos were all sacrificed. Whereas at 12 to 13 days of age the chick embryo normally weighs from 5 to 7 g, the treated embryos weighed from 0.8 to 1.2 g; the weight of a 7 day embryo. Besides the profound stunting, the abnormalities usually present were a flattened head, small eyes which frequently had a clear sac over the lens suggesting an enlarged anterior chamber, absent or underdeveloped lower beak, a long and usually twisted neck, exteriorization of the viscera, a fluid-filled sac surrounding the heart, abnormal or absent toes and various degrees of generalized edema. The abdominal organs appeared to be growing relatively more rapidly than the rest of the embryo. Occasionally, intermediate stages were seen, with larger embryos showing generalized edema, abnormal beaks, absence of feathers and limb abnormalities. Usually, however, those embryos surviving 8 to 10 days after treatment showed either the typical stunting shown in Fig. 2 or they were apparently normal. The oldest stunted embryo we have seen was 14

TABLE I.  
Distribution of C<sup>14</sup> in the Degradation Fractions from Rat Liver Glycogen, and Blood Glucose and Lactate.

Exp. No.	Animals wt in g	Total activity administered as CaC <sup>14</sup> O <sub>3</sub> in cts/min.	Activity of 7th day sample of respiratory CO <sub>2</sub> in cts/min./mg C	Activity of the various fractions from blood glucose + lactate, and liver glycogen, in cts/min./mg C				
				Blood glucose + lactate carbon atoms		Liver glycogen carbon atoms		
				3 and 4	1,2,5 and 6	3 and 4	2 and 5	1 and 6
1	328	$5.6 \times 10^6$	—	24	2	8	0	
2	303	$5.1 \times 10^6$	100	31	—	5	0	
3	293	$2.6 \times 10^7$	380	35	0	44	0	1
4	329	$2.3 \times 10^7$	640	88	0	65	0	3

in significance to that derived from the study of rat liver glycogen. The present paper reports some experiments designed to test this point in the rat.

*Methods.* Pellets of CaC<sup>14</sup>O<sub>3</sub> were implanted intraperitoneally in 4 unfasted white rats. After seven days on stock diet the animals were anaesthetized with intraperitoneal nembutal, and as much blood as possible withdrawn by syringe from the abdominal aorta. Bleeding was facilitated by administering warm Ringer's solution into the inferior vena cava by syringe. The diluted blood, rendered non-clotting by heparin, was placed in a stoppered flask, and held in a water bath at 38°C for 5 hours, with moderate agitation. At the end of this time, practically all the blood glucose was converted to lactate by the glycolytic enzymes of the blood. The blood was deproteinized with metaphosphoric acid, the lactate removed by continuous ether extraction, and degraded as previously described.<sup>1</sup> The acetaldehyde arising from the KMnO<sub>4</sub> oxidation of the lactate, representing carbons 1,2,5 and 6 of the glucose, was oxidized without further degradation. The liver glycogen was isolated and degraded by the "bacterial" degradation method described previously.<sup>1</sup> In experiments 1 and 2 the acetaldehyde arising from KMnO<sub>4</sub> oxidation of the lactate from the glycogen was precipitated and counted as the 2-4 dinitrophenylhydrazones. In experiments 3 and 4 the aldehyde was further degraded by the iodoform reaction. Daily collections of respiratory CO<sub>2</sub> were made in carbonate free NaOH.

All samples, with the 2 exceptions noted above, were converted to BaCO<sub>3</sub> and the activity estimated using an end-window G-M tube. The over-all counting error by the method in use at the time these experiments were carried out was  $\pm 4-5\%$ .

*Results and Discussion.* The experimental data is summarized in Table I. The results confirm prior findings,<sup>1,4</sup> that isotopic carbon administered as CO<sub>2</sub> appears predominantly in carbons 3 and 4 of the glucose units of liver glycogen. The distribution of isotope in the degradation fractions from blood is qualitatively similar to that found in the glycogen.

The present data must be regarded with certain reservations. The material from blood includes the blood glucose, lactate, and perhaps other related intermediates in trace amounts. The activity, as determined, might, therefore have been contributed mainly by trace substances of very high activity. In view of the fairly low levels of activity noted in the respiratory CO<sub>2</sub>, this does not seem likely. The quantitative differences between the fractions from blood and liver glycogen cannot be evaluated with certainty at present.

From the practical point of view, since the method, as applied, yielded degradation fractions from blood that mirrored those from liver glycogen, it should prove a useful tool. Additional experiments are in progress using labeled compounds known to give an isotope distribution pattern in liver glycogen different from that found in CO<sub>2</sub> fixation.

*Summary.* Experiments are reported in which it was found that CaC<sup>14</sup>O<sub>3</sub> implanted intraperitoneally in rats produced a qualitatively similar isotope distribution pattern in the liver glycogen, and blood glucose and lac-

<sup>6</sup> Armstrong, W. D., Schubert, J., and Lindenbaum, A., *Proc. Soc. Exp. Biol. and Med.*, 1948, **68**, 233.

The inability of folic acid to protect the chick embryo from the toxic action of the "4-amino" PGA's, even when given in ratios of 1000 to 4000 to 1, is further evidence of the difficulty in protecting against or reversing the activity of these compounds. Franklin *et al.*<sup>5</sup> have obtained slight and limited protection against "4-amino" PGA with large doses of folic acid, and Hertz and Tullner,<sup>15</sup> using a ratio of folic acid to 4-amino pteroylglutamic acid of approximately 300 to 1, prevented the "anti-folic" inhibition of the chick oviduct response to estrogenic stimulation. In frogs, however, a folic acid to "4-amino" PGA ratio of 100 to 1 did not prevent the "4-amino" PGA inhibition of the oviduct response to estrogens.<sup>16</sup> It appears clear, therefore, that folic acid may prevent the toxic effects of the "4-amino" folic acids but the protective ratio necessary will undoubtedly differ quantitatively for the specific "anti-folic" compound and the biological system used.

The apparently specific action of 2,6-diaminopurine on blood formation merits further

study. It is of interest that Bendich and Brown<sup>17</sup> have shown that 2,6-diaminopurine may serve as a precursor of nucleic acid guanine, an observation which cannot, as yet, be related to the mechanism of blood formation.

**Summary.** The "4-amino" pteroylglutamic acids profoundly inhibit the growth of the chick embryo, with the production of developmental abnormalities. These compounds are active in the range of 0.003 to 0.005 mg/egg, and their actions are not prevented by large doses of folic acid. The "4-amino" folic acids with aspartic acid, threonine and alanine substituted for the glutamic acid are considerably less toxic but produce similar toxicological effects. Other compounds allied to folic acid are relatively non-toxic and do not possess the growth-inhibitory activity of the "4-amino" compounds.

2,6-diaminopurine, at LD<sub>50</sub> doses, does not seem to be an active growth inhibitor, but it produces a pale embryo, presumably deficient in hemoglobin.

<sup>16</sup> Goldsmith, E. D., Schreiber, S. S., and Nigrelli, R. F., *Proc. Soc. Exp. Biol. and Med.*, 1948, **69**, 299.

<sup>17</sup> Bendich, A., and Brown, G. B., *J. Biol. Chem.*, 1948, **176**, 1471.

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## 17221. Distribution of C<sup>14</sup>, Administered as CaC<sup>14</sup>O<sub>3</sub>, in Rat Liver Glycogen and Blood Glucose and Lactate.\*

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In prior studies<sup>1-5</sup> it has been shown that the distribution of isotopic carbon in rat liver

\* Aided by a grant from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council.

<sup>1</sup> Wood, H. G., Lifson, N., and Lorber, V., *J. Biol. Chem.*, 1945, **159**, 475.

<sup>2</sup> Lifson, N., Lorber, V., Sakami, W., and Wood, H. G., *J. Biol. Chem.*, 1948, **176**, 1263.

<sup>3</sup> Lorber, V., Lifson, N., Wood, H. G., and Sakami, W., *Am. J. Physiol.*, 1948, **135**, 452.

<sup>4</sup> Shreeve, W. W., Feil, G. H., Lorber, V., and Wood, H. G., *J. Biol. Chem.*, 1949, **177**, 679.

glycogen following the feeding of a labeled compound may serve as an indicator of intermediary pathways. Under the proper experimental conditions, the distribution of isotope in blood glucose and lactate might reasonably be expected to mirror that in the liver glycogen. Should this expectation be realized, it would be possible to obtain information from the degradation of the carbohydrates from the blood of larger animals and man comparable

<sup>5</sup> Lorber, V., Cook, M., and Bordenaux, J., *Fed. Proc.*, 1949, **8**, 99.

TABLE I.  
Activation of Antifungal Activity of Butyl Alcohol Extractives of Actinomyces Culture Filtrate by Ultrafiltration.

Actinomyces No. 48240 Test material	Vol., ml	Dilution titer*	
		<i>C. albicans</i>	<i>C. neoformans</i>
Original aqueous solution of butyl alcohol extractives†	10	10	640
Ultrafiltrate	Approx. 7	160	>320
Aqueous washings	10	80	320
" ext. of membrane	5	20	80
Alcohol " " "	10	80	160
Original alcohol solution of butyl alcohol extractives†	4.5	40	640
Ultrafiltrate	Approx. 3.5	640	640
Alcohol washings	5.5	160	160
" extract of membrane	4.5	160	80

\* Highest 2-fold serial dilution to give a zone of inhibition of at least 10 mm in diffusion or cup test. Figures represent reciprocals of the dilutions.

† Aliquot of butyl alcohol extract of 1/5/49.

TABLE II.  
Activation of Antifungal Activity of Ethyl Acetate Extractives of Actinomyces Whole Culture by Gradocol Membranes.

Actinomyces No. 48240 Test material	Vol., ml	Dilution titer*	
		<i>C. albicans</i>	<i>C. neoformans</i>
Original alcohol solution	12	<5	1,280
Ultrafiltrate	12	160	20,480
Alcohol washings	12	20	1,280
" extract of membrane	6	160	320
Original sol. shaken with membrane scraps	25	10	10,240
Alcohol extr. of membrane scraps	25	<5	1,280

\* Highest 2-fold serial dilution to give a zone of inhibition of at least 10 mm in diffusion or cup test. Figures represent reciprocals of the dilutions.

ultrafiltration. For the initial concentration of active material, whole culture, culture filtrate, or pellicle was extracted with organic solvents; or culture filtrate was treated with charcoal and the charcoal eluted with organic solvents. In each case the organic solvents were removed by distillation *in vacuo* and the residue was dissolved in either water or 95% ethyl alcohol for ultrafiltration.

The effect of ultrafiltration on both a water solution and an ethyl alcohol solution of the butyl alcohol extractives from a culture filtrate of No. 48240 is shown in Table I. Not only was the activity of the ultrafiltrate much greater than that of the solution placed on the filter, but the alcohol extract of the membrane showed marked activity, especially against *C. albicans*.

In another experiment an alcohol solution of the ethyl acetate extractives of whole culture was divided into two portions; one was

passed through a gradocol membrane and the other shaken with small scraps of membrane. The results are presented in Table II. Both solutions were activated; but the activity in the one filtered through the membrane was greater, particularly against *C. albicans*. In the original solution no activity against *C. albicans* was demonstrated in a 1:5 dilution; after ultrafiltration the solution inhibited growth in a dilution of 1:160. When filtrates of the broth cultures of the actinomycetes were treated with charcoal (Darco G 60) to remove the active agent and the charcoal was eluted with water or organic solvents, no activity against *C. albicans* could be demonstrated by diffusion tests. Ultrafiltration, however, resulted in activation of these eluates against *C. albicans* to a dilution of 1:640. The protocol of such an experiment is given in Table III.

Enhancement of activity was not peculiar



tate. This result suggests that in isotope feeding experiments, analysis of the blood glucose and lactate may yield information comparable to that obtained by the degradation of liver glycogen. Additional experi-

ments to put this question to a more critical test are in progress.

It is a pleasure to thank Miss Margaret Cook for valuable technical assistance.

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## 17222. Activation of Antifungal Extracts of Actinomycetes by Ultrafiltration Through Gradocol Membranes.

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In the course of the purification of extracts from broth cultures of two soil actinomycetes showing antifungal activity, it was observed that solutions which were passed through gradocol membranes often possessed greater activity than the original solutions. Striking results were obtained repeatedly and under varying conditions. Since there may be a wide application in studies of antibiotics, it seems well to report the original observations. The extent and nature of the phenomenon are under investigation.

**Materials and methods. Microorganisms.** Two distinct actinomycetes, Nos. 47205<sup>1</sup> and 48240, isolated from soil and found to be antagonistic to fungi pathogenic for man, were grown in liquid media dispensed in 250-ml amounts in 1000-ml Erlenmeyer flasks. Strain No. 47205 was grown in glycerol-yeast extract broth containing 0.15% agar for from 9 to 12 days at approximately 28°C, and No. 48240 in glucose-tryptone broth for from 12 to 17 days at the same temperature. The composition of the media varied from the original formulas<sup>1</sup> only in that distilled water replaced the tap water. Four pathogenic fungi, *Candida albicans* No. 4567, *Cryptococcus neoformans* No. 45215, *Trichophyton mentagrophytes* (gypseum) No. 45141, and *Trichophyton rubrum* (purpureum) No. 4507 were employed as test microorganisms.

**Antifungal tests.** The extracts were assayed by the agar-diffusion or cup test and

by agar-streak or agar-dilution tests.<sup>2</sup> Glucose-tryptone agar<sup>1</sup> was used throughout. The time of incubation varied according to the rate of growth of the particular test microorganism and the type of test. Thus in the diffusion tests in which *C. albicans* was employed, incubation for 8 to 10 hours was adequate, while with *C. neoformans*, 22 to 24 hours were necessary. The agar-streak tests with all 4 test microorganisms were incubated 72 hours. All tests were incubated at approximately 28°C.

**Ultrafiltration.** Gradocol membranes were prepared from nitrocellulose in the form of Parlodion according to the method described by Bauer and Hughes.<sup>3</sup> The average pore diameter of the several membranes varied from 52 to 64 mμ.\* The solutions were passed through the membranes under positive pressure. After filtration, the membranes were washed by passing through some of the pure solvent. They were then removed from the filter and extracted with a small volume of 95% ethyl alcohol. Whenever the pH of a solution was determined before ultrafiltration, it was in the acid range.

**Experimental.** Different types of solutions showing antifungal activity were subjected to

<sup>2</sup> Reilly, H. C., Schatz, A., and Waksman, S. A., *J. Bact.*, 1945, 49, 585.

<sup>3</sup> Bauer, J. H., and Hughes, T. P., *J. Gen. Physiol.*, 1934, 18, 143.

\* In one experiment with a coarse membrane, 378 mμ, ultrafiltration did not result in activation of the solution.

<sup>1</sup> Schatz, A., and Hazen, E. L., *Mycologia*, 1948, 50, 461.

TABLE V.  
Activation of Antifungal Activity by Ultrafiltration Demonstrated by Agar-streak (Agar-dilution) Tests.

Dilution of material	Growth of test fungi							
	<i>C. albicans</i>		<i>C. neoformans</i>		<i>T. gypsum</i>		<i>T. purpureum</i>	
	Original*	Ultra-filtered	Original*	Ultra-filtered	Original*	Ultra-filtered	Original*	Ultra-filtered
1:40	4+	—	—	—	—	—	—	—
1:80	4+	—	—	—	±	—	±	—
1:160	4+	—	—	—	2+	—	2+	—
1:320	4+	—	—	—	3+	—	3+	—
1:640	4+	—	—	—	3+	—	3+	—
1:1,280	4+	2+	±	—	3+	—	3+	±
1:2,560		3+		2+		—		±
Controls	4+		4+		4+		4+	

\* The ethyl alcohol solution of ethyl acetate extractives from No. 48240 culture filtrate.

— = No visible growth.

± = Least detectable growth.

+ = Scanty, but definite growth.

2+ = Moderate growth.

3+ = Good growth.

4+ = Maximum growth.

hand, Melin and Wikén<sup>8</sup> reported that aqueous extracts of pure forest litter that were somewhat active against staphylococci became more active when autoclaved or passed through a Seitz filter pad.

Whatever the explanation of the present results, it is evident that the ultrafiltration

technic may be applied profitably in the study of antibiotics.

**Summary.** The antifungal activity of some partially purified extracts of two actinomycetes was sharply increased by filtration through gradocol membranes.

We wish to thank Mr. James Quigley for carrying out all the procedures in ultrafiltration.

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### 17223. Relation of a Specific Strain of Salmonella to Ulcerative Cecitis of Rats.

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Although *Salmonella enteritidis* is found very constantly in the lesions of ulcerative cecitis of rats<sup>1</sup> some doubt remains as to whether this organism is the sole cause of the disease. The possibility that some other agent, perhaps a virus, may be concerned is suggested by certain findings; the arguments pro and con can be summarized briefly as follows:

(1) *Arguments in favor of Salmonella being the specific etiological agent in cecitis of rats.* a. *Salmonella enteritidis* has been found by all workers to be invariably associated with the lesions.<sup>2</sup> b. *Salmonella* may be obtained from the enlarged mesenteric lymph nodes associated with cecitis.<sup>2</sup> c. Cecitis may be prevented by rations containing either sulfaguanidine or sulfasuxidine.<sup>3</sup> d. The le-

<sup>1</sup> Buchbinder, L., Hall, L., Wilens, S. L., and Slanetz, C. A., *Am. J. Hyg.*, 1935, 22, 119.

<sup>2</sup> Bloomfield, A. L., and Lew, W., *Proc. Soc. Exp. Biol. and Med.*, 1942, 51, 179.

TABLE III.

Activation of Antifungal Activity of Charcoal Eluates of Actinomycete Culture Filtrate by Ultrafiltration.

Actinomycete No. 48240 Test material	Vol., ml.	Dilution titer*	
		<i>C. albicans</i>	<i>C. neoformans</i>
Original alcohol solution of charcoal eluates	10	0	80
Ultrafiltrate	9	640	640
Alcohol washings	10	40	40
" extr. of membrane	5	320	320

\* Highest 2-fold serial dilution to give a zone of inhibition of at least 10 mm in diffusion or cup test. Figures represent reciprocals of the dilutions.

TABLE IV.

Activation of Antifungal Activity of Extractives from Actinomycete Culture Pellicle by Ultrafiltration.

Actinomycete No. 47205 Test material	Vol., ml.	Dilution titer*	
		<i>C. albicans</i>	<i>C. neoformans</i>
Original alcohol solution	10	<5	>320
Ultrafiltrate	Approx. 7.5	320	640
Alcohol washings	10	40	160
" extr. of membrane	5	160	160

\* Highest 2-fold serial dilution to give a zone of inhibition of at least 10 mm in diffusion or cup test. Figures represent reciprocals of the dilutions.

to a single actinomycete nor to one culture medium. Similar results obtained with No. 47205 are shown in Table IV. In this case extractives obtained from the pellicle with ethyl acetate were chromatographed on alumina and an alcoholic eluate subjected to ultrafiltration. The original solution was without activity against *C. albicans* but the ultrafiltrate inhibited growth in a dilution of 1:320. By the agar-streak test enhancement of antifungal activity of the ethyl acetate extractives of No. 48240 culture filtrate was also demonstrated against *C. albicans*, *C. neoformans*, *T. gypseum*, and *T. purpureum*. (Table V).

Water or alcohol ultrafiltrates of gradocol membranes are inactive indicating that the phenomenon is not due to a principle in the filter itself.

**Discussion.** It is not uncommon for the activity of antibiotic substances to be reduced or lost unaccountably during the course of purification. A few such observations that seem to be more or less related to our own

are noted briefly. Woodruff and Foster<sup>4</sup> traced the loss of antibacterial activity in bacillin to the presence of an inhibitor of peptide nature that occurs naturally in many complex organic substances. Cavallito and others<sup>5</sup> found that sulphydryl compounds had an inactivating effect upon some antibiotics. In an examination of the separation of penicillins by the method of counter-current distribution, Craig and his colleagues<sup>6</sup> observed that the use of ethyl acetate led to a certain degree of inactivation. They suggested that this might be due to a particular lot of ethyl acetate. Another observation that may be related was made by Junowicz-Kocholaty and others:<sup>7</sup> namely, that in the purification of surfactin, activity apparently disappeared in butyl alcohol extracts, but reappeared subsequently in chloroform extracts. On the other

<sup>5</sup> Cavallito, C. J., Bailey, J. H., Haskell, T. H., McCormick, J. R., and Warner, W. F., *J. Bact.*, 1945, 50, 61.

<sup>6</sup> Craig, L. C., Hogeboom, G. H., Carpenter, F. H., and duVigneaud, V., *J. Biol. Chem.*, 1947, 168, 665.

<sup>7</sup> Junowicz-Kocholaty, R., Kocholaty, W., and Kelner, A., *J. Biol. Chem.*, 1947, 168, 765.

<sup>4</sup> Woodruff, H. B., and Foster, J. W., *J. Bact.*, 1946, 51, 371.

TABLE V.

Activation of Antifungal Activity by Ultrafiltration Demonstrated by Agar-streak (Agar-dilution) Tests.

Dilution of material	Growth of test fungi							
	<i>C. albicans</i>		<i>C. neoformans</i>		<i>T. gypsum</i>		<i>T. purpurcum</i>	
	Original*	Ultra-filtered	Original*	Ultra-filtered	Original*	Ultra-filtered	Original*	Ultra-filtered
1:40	4+	—	—	—	—	—	—	—
1:80	4+	—	—	—	±	—	±	—
1:160	4+	—	—	—	2+	—	2+	—
1:320	4+	—	—	—	3+	—	3+	—
1:640	4+	—	—	—	3+	—	3+	—
1:1,280	4+	2+	±	—	3+	—	3+	±
1:2,560		3+		2+		—		±
Controls	4+		4+		4+		4+	

\* The ethyl alcohol solution of ethyl acetate extractives from No. 48240 culture filtrate.

— = No visible growth.

± = Least detectable growth.

+ = Scanty, but definite growth.

2+ = Moderate growth.

3+ = Good growth.

4+ = Maximum growth.

hand, Melin and Wikén<sup>8</sup> reported that aqueous extracts of pure forest litter that were somewhat active against staphylococci became more active when autoclaved or passed through a Seitz filter pad.

Whatever the explanation of the present results, it is evident that the ultrafiltration

technic may be applied profitably in the study of antibiotics.

**Summary.** The antifungal activity of some partially purified extracts of two actinomycetes was sharply increased by filtration through gradocol membranes.

We wish to thank Mr. James Quigley for carrying out all the procedures in ultrafiltration.

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### 17223. Relation of a Specific Strain of Salmonella to Ulcerative Cecitis of Rats.

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Although *Salmonella enteritidis* is found very constantly in the lesions of ulcerative cecitis of rats<sup>1</sup> some doubt remains as to whether this organism is the sole cause of the disease. The possibility that some other agent, perhaps a virus, may be concerned is suggested by certain findings; the arguments pro and con can be summarized briefly as follows:

(1) *Arguments in favor of Salmonella being the specific etiological agent in cecitis of rats.* a. *Salmonella enteritidis* has been found by all workers to be invariably associated with the lesions.<sup>2</sup> b. *Salmonella* may be obtained from the enlarged mesenteric lymph nodes associated with cecitis.<sup>2</sup> c. Cecitis may be prevented by rations containing either sulfaguanidine or sulfasuxidine.<sup>3</sup> d. The le-

<sup>1</sup> Buchbinder, L., Hall, L., Wilens, S. L., and Slanetz, C. A., *Am. J. Hyg.*, 1935, 22, 119.

<sup>2</sup> Bloomfield, A. L., and Lew, W., *Proc. Soc. Exp. Biol. and Med.*, 1942, 51, 179.

sions can be cured by ingestion of streptomycin which inhibits (*in vitro*) in high dilution the strains of salmonella recovered from the cecum.<sup>4</sup> e. Attempts to isolate a virus have so far been fruitless.<sup>5</sup>

(2) *Arguments which suggest that some other agent may play a part.* a. The anatomy of the lesions—early involvement of lymph nodes and later localized granulomatous lesions are not known to be caused by members of the salmonella group. b. The large cysts containing clear fluid so often found as part of the cecitis picture do not yield salmonella or other organisms by ordinary culture technic.<sup>2</sup> c. Salmonella isolated from the lesions and fed in large dosage to healthy rats produce a violent diffuse enteritis, rather than the usual picture of cecitis.<sup>2</sup> d. Some of the animals which recovered from such an acute enteritis were later found to have typical lesions of chronic cecitis but with no greater frequency than uninoculated controls.<sup>2</sup> e. Cecitis in the offspring can be prevented by treatment of the mothers with sulfonamides during pregnancy and lactation. It is not clear why such a procedure should protect against salmonella infection.<sup>6</sup> f. Rats fed a diet deficient in Vitamin B develop cecitis less often than controls given a normal diet.<sup>7</sup> This result suggests the possibility of a virus infection and is just the opposite of what one would expect with salmonella.

*Experiments.* The following studies were therefore made in the attempt to define more precisely the role of salmonella in chronic ulcerative cecitis of rats. The first group of experiments was designed to determine relation of the development of lesions to the presence of salmonella and to the titer of specific antibodies.

Seven groups of 6 young rats were used. Each group was housed in a separate cage

<sup>3</sup> Bloomfield, A. L., and Lew, W., *ibid.*, 1941, 48, 363; 1942, 51, 28.

<sup>4</sup> Bloomfield, A. L., and Lew, W., *ibid.*, 1948, 69, 11.

<sup>5</sup> Schultz, E. W., personal communication.

<sup>6</sup> Bloomfield, A. L., and Lew, W., *Am. J. Med. Sci.*, 1943, 205, 383.

<sup>7</sup> Bloomfield, A. L., and Lew, W., *J. Nutrition*, 1943, 25, 427.

and was fed the stock diet of the laboratory as desired. At the age of 2 months and thereafter at intervals of 2 weeks one group was sacrificed. Under ether anaesthesia and with aseptic technic the abdomen was opened and the cecal region was carefully examined for suspicious lesions. The cecum was then excised, opened, and searched for ulceration. Material for culture was taken from the cecum, the swab being thoroughly rubbed over the mucosa. Except in the case of the first 2 groups the animals were then exsanguinated by cutting the abdominal vessels from which blood was collected for serological tests.

Stool cultures were made on MacConkey (Difco) and salmonella-shigella (Difco) agar plates, from which colonies were sub-cultured in Russell double sugar tubes and in liquid carbohydrate media. Various strains were finally tested with salmonella diagnostic sera (Lederle). Sera to be tested for agglutinins were stored in the refrigerator. Serial dilutions were set up against a live suspension of 24 hour growth of the salmonella isolated from cecal lesions. The tubes were held for 2 hours in the water bath at 37°C placed over-night in the refrigerator and then read at room temperature.

*Results.* The results are summarized in Table I. Aside from the normal bowel flora the only organisms isolated were members of the salmonella group. These were obtained from 9 animals among a total of forty-two. In each case the characteristics were identical and conformed to *Salmonella enteritidis*, Group D of the Kauffmann-White Schema. Furthermore 6 strains which were identified in the salmonella typing laboratory of the State Department of Public Health all had the same antigenic formula: — (I), IX, XII . . . : g . . . ; —

A study of Table I shows several points of interest. There was no visible cecitis in the 2 to 3 months old groups. From 3½ months on, with the exception of the 4 months group, cecitis was evident in approximately one-half the animals. Organisms of the salmonella group were recovered in only one instance in which gross lesions were not evident, (Rat I of Group IV), and then only

TABLE I.  
Relation of Lesions of Cecitis to Presence of Salmonella, and Titer of Agglutinins in Rats of Various Ages.

Rat. No.	Lesions	Culture Salmonella	Agg. titer
Group I—2 mo.			
6 rats	None	All negative	Not done
Group II—2½ mo.			
6 rats	None	All negative	Not done
Group III—3 mo.			
6 rats	None	All negative	4, 0, 0, 0, 4, 8
Group IV—3½ mo.			
Rat No. 1	No cecitis	10	32
2	Walls of cecum seem a little thick, no ulcer	50	8
3	No ulcer seen but wall a little thick and there is a mesenteric cyst 0.5 cm	10	32
4	No cecitis	0	0
5	Frank cecitis with ulcer 1 cm in diam.	Innumerable	32
6	No cecitis	0	0
Group V—4 mo.			
6 rats	None	All negative	8, 4, 4, 4, 4, 0
Group VI—4½ mo.			
Rat No. 1	No cecitis	Negative	8
2	" "	" "	8
3	Marked thickening of cecum with ulcer 0.5 cm, large cysts and adhesions	Many	16
4	No cecitis	Negative	32
5	" "	" "	64
6	Typical ulcer 0.5 cm	Many	8
Group VII—5 mo.			
Rat No. 1	No ulcer, thickening of cecal wall and cyst 0.5 cm	A few	32
2	Extreme lesions, huge ulcer	Innumerable	32
3	No cecitis seen	Negative	8
4	" " "	" "	16
5	Marked lesion, ulcer 1 cm	Innumerable	16
6	No cecitis seen	Negative	0

a few colonies were obtained. The number of salmonella usually paralleled the extent of the lesions; when the lesion was slight a few up to 50 organisms per plate were obtained, when the lesion was advanced there were always "many" to "innumerable" colonies. The point of greatest importance however, is that as rats grow and develop cecitis salmonella are already present in the earliest lesions; they do not appear only after cecitis is advanced.

The agglutinins are of special interest. Among the 8 animals with visible cecitis 2 had a titer of 8, two had a titer of 16, and 4

had a titer of 32. Among 21 animals without visible lesions and with negative cultures for salmonella, however, 14 showed titers varying from 4 to 64. Furthermore, one-half the animals in the 3 month old group showed agglutinins at a time when no cecitis was yet to be seen and no cultures were positive.

It is clear then that there is an early and frequent, although slight, immunological reaction to salmonella enteritidis in these rats at a time before lesions of cecitis are visible, and before it is possible to isolate salmonella by our technic. The question was pursued further by examining the sera from even younger

TABLE II.  
Agglutinin Titer Against *Salmonella enteritidis* of Young Healthy Rats.

Age 2 months		Age 1 month.		Age 12 days	
Rat No.	Titer	Rat No.	Titer	Rat No.	Titer
2A-F	8, 4, 16, 4, 8, 16	1A-F	8, 8, 32, 8, 8, 4	1-6 Mother	0, 4, 0, 4, 8, 8

rats. All the animals were healthy and none showed cecitis. Six were 2 months old, 6 were one month old, and 6 were 12 days old. The eyes were not yet open in the last group and they had not yet been weaned. Serum from the mother of this last group was also tested. The results are shown in Table II. It is seen that even the younger rats may already show specific agglutinins against salmonella at a time long before cecitis can be demonstrated or salmonella isolated. The presence of such agglutinins are therefore of little diagnostic significance and it will be of interest to look for similar antibodies in a rat colony entirely free from cecitis.

Further evidence for the causal role of salmonella was then sought in the following experiments. Sixty rats about 2 months old were divided into 3 groups of 10 test animals and three groups of litter-mate controls. On 5 consecutive days one solid loop of growth from a 24 hour agar slant of salmonella enteritidis isolated from rats with cecitis was shaken up in the water bottles of the test animals. Culture of the water thereupon showed heavy growth. None of the animals however, developed any clinical indisposition with these doses and they all gained weight normally. After one, 2, and 3 months when the rats were 3, 4, and 5 months old one set of test animals with its controls were sacrificed under ether anaesthesia and the degree of cecitis, if any, was noted. The results are shown in Table III. It is seen that one month after ingestion of salmonella there was very little cecitis in either test animals or controls. After 2 months definite cecitis was present in some animals, a little more in the test group than in the controls; but after 3 months cecitis was rampant in the culture-fed animals with not a single definite lesion in the controls.

*Discussion.* The above experiments were designed to define more exactly than has here-

fore been done the significance of *Salmonella enteritidis* in cecitis of rats. The invariable relationship of salmonella to the lesions was confirmed; no culture from a visible lesion, even very early, failed to yield the specific organism. On the other hand salmonella were never recovered from the cecum unless a lesion was present except in one instance (animal No. 1 of the 3½ months group) and then only a few colonies were isolated. Specific agglutinins were uniformly present in the sera from animals with cecitis, but since such agglutinins were frequently present without either cecitis or a positive culture for salmonella, the significance of these antibodies is uncertain. On the whole higher titers were obtained from animals with frank lesions than from healthy rats.

These observations demonstrate conclusively that no cecitis occurs in this colony without the presence of the specific salmonella. However, this finding does not entirely exclude the association of some other unidentified agent in producing the infection. The inoculation experiments are of interest in this connection. Rats fed salmonella did in the end show much more cecitis than untreated controls but the lesions developed only after what appeared to be a long latent period of from one to 3 months. Where are the specific bacteria during this long interval? There is some suggestion that the earliest lesion of the cecitis syndrome is an adenitis of the regional nodes, (Table III) and that the actual cecal lesion only develops after a considerable interval. If this is true the long latent period may be explained, but such a course of events is unknown with salmonella infections. We plan to explore this possibility in further studies. Meanwhile it seems clearly established that ingestion of the specific salmonella promoted the development of cecitis which in turn never occurred in the absence of the

TABLE III.  
Incidence of Cecitis in Rats Fed *Salmonella* Enteritidis, and in Untreated Controls.

Degree of cecitis in rats aged 3 mo.		Degree of cecitis in rats aged 4 mo.		Degree of cecitis in rats aged 5 mo.	
Test animals	Controls	Test animals	Controls	Test animals	Controls
0	0	0	0	0	0
0	0	0	0	0	0
0	0	0	0	0	0
0	0	0	0	?	0
?	0	0	0	++	0
?	0	?	0	+++	0
?	0	?	0	+++	0
?	0	++	0	+++	0
+	0	++	?	++++	0
+	?	++	++++	++++	?

0 = No ulcer, no glands, no cysts.

? = No ulcer, slight or doubtful enlargement of glands, no cysts.

+ = Ulcer to 0.5 cm, enlarged glands and/or cysts.

++ = Ulcer 0.5 to 1.0 cm, enlarged glands and/or cysts.

+++ = Ulcer 1.0 to 2.0 cm, enlarged glands, cysts, adhesions, and matting.

++++ = Huge ulcers, enlarged glands, cysts, adhesions, and matting.

organisms. Of special interest is the fact that in spite of the extensive infestation of the colony healthy carriers are of greatest rarity.

**Summary.** 1. A specific strain of salmonella has been invariably obtained on culture from the ulcers of rat cecitis. 2. *Salmonella* were not obtained from the cecum in the absence of lesions with one exception. 3. Agglutinins for the specific strain of salmonella

are found in most of the rats in this colony although the titer is on the average higher in animals with visible cecitis. 4. Feeding the specific salmonella in appropriate doses was followed after a long latent period by development of cecitis in many of the test animals. 5. The association of some other synergistic agent is not fully excluded but seems at present unlikely.

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## 17224. Aminopterin and Response of Frog Oviducts to Estradiol. II. Histological Studies and Mitotic Counts.\*

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In a previous paper,<sup>1</sup> the gross changes in the immature female frog oviduct following parenteral administration of estradiol benzoate<sup>†</sup> alone and in conjunction with folic acid<sup>‡</sup> and with a folic acid antagonist, amin-

opterine,<sup>‡</sup> were described. Aminopterin (4-amino pteroylglutamic acid) inhibited the growth and differentiation response of oviducts of newly metamorphosed female frogs, *Rana clamitans*, to estradiol. The aminopterin-

\* This investigation was supported by a research grant from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service.

<sup>1</sup> Goldsmith, E. D., Schreiber, S. S., and Nigrelli, R. F., *Proc. Soc. Exp. Biol. and Med.*, 1948, 69, 299.

<sup>†</sup> The estradiol benzoate was generously supplied by Dr. F. F. Yonkman and Dr. F. L. Mohr of Ciba Pharmaceutical Products.

<sup>‡</sup> The 4-amino pteroylglutamic acid was generously supplied by the late Dr. Y. Subbarow, and the folic acid by Dr. T. H. Jukes and the late Dr. A. L. Franklin of Lederle Laboratories.



in effect could not be overcome by folic acid in concentrations as high as 100 parts of folic acid to 1 part of aminopterin. In the light of more recent work, it is probable that pre- and concurrent treatment with higher dosages of folic acid would have reversed the antagonistic action.

*Animals were fixed in Bouin's solution and serial sections of oviducts extending along the craniad half or two-thirds of the kidney were cut at 7  $\mu$  and were stained with hematoxylin and eosin, iron hematoxylin, or with Masson's stain. Mitotic counts were made of the oviduct epithelium; sections from each slide were examined so that comparable areas in all the animals were studied in order to obtain estimates of mitotic activity throughout the length of the oviduct.*

*Results.* In the untreated controls, the small and uncoiled oviducts consisted of a single layer of cuboidal epithelium with regular oval or round nuclei which encircled a small lumen. Mitotic counts of the oviduct epithelium revealed less than one mitotic figure per thousand cells. After the administration of estradiol for 2 to 3 weeks (0.1 mg per week), there was marked increase in the diameter, length and coiling of the oviduct. The bulk of the oviducts consisted of glands which radiated from the oviduct lumina to the periphery. The gland cells contained basal nuclei and at times granular cytoplasm. The lumina were lined with well differentiated ciliated columnar epithelium with regular oval, vesicular nuclei. Mitotic figures were rare, and in over 12,000 cells the incidence of mitotic figures was less than one per 1500 cells. Addition of folic acid to estradiol accentuated the growth and differentiation response of the oviducts, but the mitotic counts were not significantly different from those animals receiving only estradiol.

Either pretreatment with aminopterin or simultaneous treatment of the frogs with aminopterin and estradiol appeared to inhibit the estrogen response. The oviducts remained uncoiled, increased in diameter only slightly and showed areas of cystic dilatation. There was no evidence of gland formation, and a single layer of low columnar epithelium lined the lumina which were only slightly larger

than those in untreated controls. In the areas of cystic dilatation, the epithelium around the lumina was flat and almost squamous in appearance. There was a significant increase in mitotic figures in these oviducts, with 12 to 36 figures per thousand cells. Although metaphases predominated, there were considerable numbers of the other mitotic phases.

Except for some slightly increased infolding of the oviduct epithelium into the lumina, supplementation of the aminopterin and estradiol with folic acid did not significantly change the histological picture characteristic of those receiving only the antagonist and estradiol. Mitotic counts in all but one animal revealed 17 to 46 mitotic figures per thousand cells. In 2 animals, one receiving aminopterin and estradiol, and the second receiving folic acid as well, where there was no significant increase in mitoses over the controls, the nuclei of the oviduct epithelium were markedly abnormal, with great variations in size and shape. The rest of the histological picture was no different from other animals treated similarly.

*Discussion.* The results indicate that although the growth and differentiation of the oviducts were inhibited by aminopterin the number of mitotic figures was significantly increased. It must be emphasized that the administration of estradiol alone is followed by an increase in the number of cells and mitoses. However, at the time of sacrifice, 2 to 3 weeks after initiation of treatment, the most rapid growth had passed its peak. At the end of this period, the oviducts under the influence of the antagonist and the estrogen had grown but slightly, but a large number of mitotic figures were present.

This apparently paradoxical situation of a picture of numerous mitotic figures and very little growth may be resolved by the following considerations.

From the observations of Brachet<sup>2</sup> and Painter,<sup>3</sup> Koller<sup>4</sup> has adduced that the amount of available nucleic acid is one of the principal factors which determine the rate of cell di-

<sup>2</sup> Brachet, J., *Arch. Biol., Paris*, 1940, **51**, 151.

<sup>3</sup> Painter, T. S., *Proc. Nat. Acad. Sci.*, 1940, **26**, 95.

<sup>4</sup> Koller, P. C., *Nucleic Acid*, Cambridge Univ. Press, 1947, p. 280.

vision. It was suggested<sup>5</sup> that folic acid may function as a coenzyme in a system responsible for the synthesis of thymine or a thymine-like compound which is then utilized in the synthesis of nucleic acid. Further evidence of this relationship is to be seen in *Lactobacillus casei* which when grown in a folic acid deficient medium showed a marked decrease in desoxyribonucleic acid,<sup>6</sup> a universal constituent of cell nuclei.<sup>7</sup>

It is tempting to speculate that in the presence of aminopterin, a folic acid antagonist, the folic acid utilization in the organism may be interfered with, resulting in a reduction in nucleic acid synthesis and a marked retardation of the rate of cell division. These facts might serve to explain the growth inhibition obtained with the folic acid antagonists in

certain of the leukemias<sup>8,9</sup> and in the transplantable mouse Sarcoma 180.<sup>10</sup>

**Summary.** Macroscopically and microscopically, oviducts of frogs receiving aminopterin (4-amino pteroylglutamic acid) failed to respond to the growth stimulating action of estradiol. However, a significantly greater number of mitotic figures were visible in these oviducts. Evidence is presented to suggest that the folic acid antagonists may exert their growth inhibitory action by interfering with folic acid utilization in the organism, with a consequent reduction in nucleic acid synthesis and a retardation of the rate of cell division.

<sup>8</sup> Farber, S., Diamond, L. K., Mercer, R. D., Sylvester, R. F. Jr., and Wolff, J. A., *New England J. Med.*, 1948, **238**, 787.

<sup>9</sup> Burchenal, J. H., Burchenal, J. R., Kushida, M. N., Johnston, S. F., and Williams, B. S., *Cancer*, 1949, **2**, 113.

<sup>10</sup> Moore, H. E., Stock, C. C., Sugiura, K., and Rhoads, C. P., *PROC. SOC. EXP. BIOL. AND MED.*, 1949, **70**, 396.

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<sup>5</sup> Stokes, J. L., *J. Bact.*, 1944, **48**, 201.

<sup>6</sup> Prusoff, W. H., Tepley, L. J., and King, C. G., *J. Biol. Chem.*, 1948, **176**, 1309.

<sup>7</sup> Stedman, E., and Stedman, E., *Nucleic Acid*, Cambridge Univ. Press, 1947, p. 232.

## 17225. Increased Metabolic Rate in Rats after X-Irradiation.

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Some metabolic reactions may be increased after exposure to total-body irradiation. Indirect evidence for increased metabolism in dogs 1-3 weeks after irradiation was presented by Prosser *et al.*:<sup>1</sup> (1) Weight loss was greater after irradiation than in non-irradiated dogs in which food intake was limited to amounts less than consumed by the irradiated animals. (2) Nitrogen excretion was maintained by irradiated dogs despite a decrease in food intake, and in the acute terminal period the nitrogen balance was negative. (3) The metabolic balance minus the

water balance or metabolic index is given by  $\{(\text{food} + \text{water} + \text{weight loss}) - (\text{urine} + \text{feces})\} - \{(\text{food water} + \text{water drunk}) - (\text{urine} + \text{feces water})\}$ . This metabolic index is a measure of CO<sub>2</sub> exhaled plus water of metabolism and water of tissue breakdown. This quantity increased significantly in dogs during the first 2 weeks after irradiation and increased considerably during the acute terminal period.

As a check on the hypothesis that catabolism is increased after irradiation, basal metabolism has been measured in rats after total body exposure to x-rays.

**Materials and methods.** Forty-four albino rats, 24 males and 20 females, ranging in initial weight from 160 to 310 g, (average 206 g) were used. There was no apparent dif-

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<sup>1</sup> Prosser, C. L., with contributions by Painter, E. E., Lisco, H., Brues, A. M., Jacobson, L. O., and Swift, M. N., *Radiology*, 1947, **49**, 299.

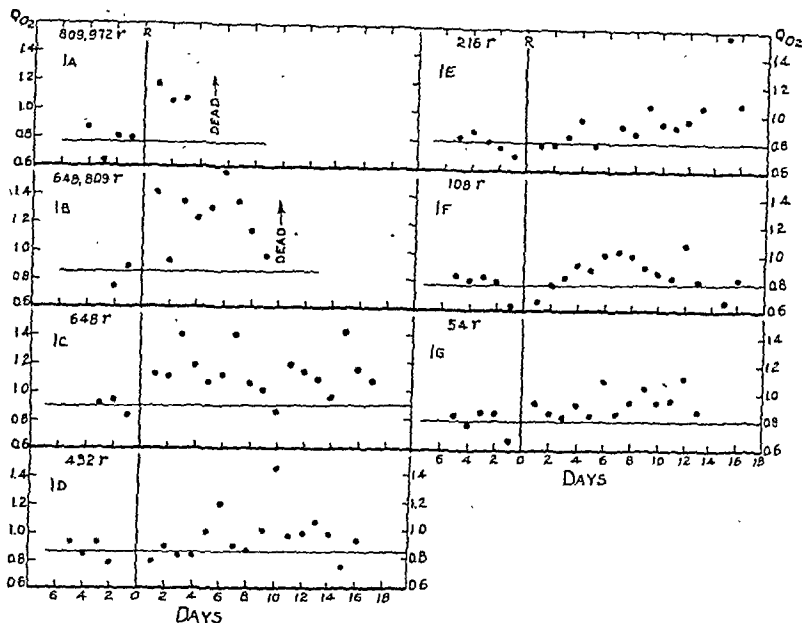


FIG. 1a-g.

Average oxygen consumption in ml  $O_2$ /g/hr of groups of rats irradiated at day 0. 1a: 6 rats which died 3-5 days after exposure; 1b: 8 rats which died 8-10 days after exposure; 1c: 6 rats which survived potentially lethal doses; 1d: 4 rats exposed to 432 r. 1e: 5 at 216 r. 1f: 5 at 108 r. 1g: 4 at 54 r. Horizontal lines: Control average  $Q_{O_2}$  for each group.

ference in reaction as a function of size or sex. The postabsorptive oxygen consumption was measured daily between noon and 4 P.M.; animals were weighed after each metabolism measurement.

The equipment used for measurement of oxygen consumption was a modification of the apparatus described by Schwabe and Griffith.<sup>2</sup> This is a closed gas circuit consisting of a glass animal chamber, a circulating oil-immersed pump,  $CO_2$  and  $H_2O$  absorbers, and two flasks arranged as water valves which maintain a constant pressure in the entire system. Oxygen is supplied through the pressure-maintaining flasks from a rubber bag. Volume differences in the first of the pressure flasks are transmitted to a water manometer from which a float writes on a kymograph. Movement of the animal was reflected in a steeper rise of the kymograph record. All measurements of  $Q_{O_2}$  were based on the least slope, (which corresponded to periods when the animal was at

rest) and were expressed as ml of  $O_2$  consumed per g body weight per hour. Animals were placed in the chamber for a half hour each day until they became accustomed to the apparatus. Then control measurements were made for several days prior to irradiation.

The rats were irradiated, 2 to 3 at a time, in a cell cut into a block of presswood. The dimensions of the block were 40 x 40 x 20 cm, those of the cell 18 x 18 x 10 cm. One half of the dose was administered from above, one half from below. Factors were: 200 K.V.p., no filter, T. D. of 55½ cm (to center of cell). The dosage distribution was determined by measurement with a Victoreen thimble chamber in a phantom of presswood equal in size to the irradiation block and cell. It was found that the dosage distribution was fairly uniform, the minimal dose (at the corners of the cell) being 83% of the maximal dose (in the center). The average tissue dose rate was 40.5 r per minute.

*Experimental results.* The animals have been divided into 4 groups. in a way that was

<sup>2</sup> Schwabe, E. L., and Griffith, F. R., *J. Nutrition*, 1938, 15, 127.

TABLE I.  
Oxygen Consumption in Control Animals: Analysis of Variance.

	Sum of squared deviations	Degrees of freedom	Variance	Standard deviation
Daily variations	0.39	55	0.007	0.08
Var. between rats	0.35	17	0.021	0.14
Total variation	0.74	72	$\delta 1$	
about the general mean = 81			$\delta 2$	
			1.75; P < 0.0027	

suggested by a survey of the responses obtained. Of 20 rats which received doses of from 648 to 972 r, 6 died 3 to 5 days, 8 died 9-10 days after treatment, and the remaining 6 survived. The clustering of survival times around 2 values suggests 2 groups defined by survival times. That deaths occur at certain times after irradiation has been frequently observed; the clinical syndrome is different according to the time of death. The third group contains the rats which survived a potentially lethal dose. In the fourth group are animals treated with sublethal doses. The group averages of  $Q_{O_2}$  values are presented in Fig. 1.

**Controls.** Measurements of oxygen consumption before irradiation yielded an average of 0.83 ml/g/hr which is slightly higher than values given by Moses.<sup>3</sup> The variance was analyzed, following R. A. Fisher's procedure,<sup>4</sup> on the material obtained from 17 rats on each of which 4 to 5 control measurements had been made. The data are given in Table I which shows that the oxygen consumption of any single rat varies from day to day with a standard deviation of about 10% of the mean, and that differences between rats are higher than expected on the basis of random sampling. The excess standard deviation is about 15% of the mean.

**Death in 3 to 5 days.** Six of 12 rats which received 972 or 809 r developed severe diarrhea, lost weight rapidly, and died 3 to 5 days after exposure. Their  $Q_{O_2}$  was increased by about 35% within 24 hours after treatment (earlier measurements were not taken) (Fig.

1a). These animals lost an average of 17% of body weight by the time of death (Fig. 2a).

**Death in 9 to 10 days.** Six of the 12 rats irradiated with 809 or 972 r, and 2 out of 8 treated with 648 r, died 9 to 10 days after irradiation. No severe diarrhea occurred in this group. Their  $Q_{O_2}$  was elevated at the first measurement after exposure and remained—on an average—58% higher than the preirradiation level until the day before death, when it declined (Fig. 1b). Weight loss was not rapid in this group; it reached 17% at 5-6 days (Fig. 2b). Thereafter, there was little change in weight, some individuals showing a slight gain. Weight loss definitely did not mirror the course of the metabolic rate.

**Survival after potentially lethal doses.** Six of 8 rats which received 648 r survived beyond the time when acute death occurs. Each of these showed an increase in oxygen consumption. The average increase was 32% during the first 7 days. During the period of 8 to 10 days after the irradiation—at the time when the second group of non-survivors was dying—there was in some individuals a depression of the metabolic rate to only 10% above the preirradiation level. After this brief period, higher  $Q_{O_2}$  values (on the average 25% above normal) were again obtained. At 17 days, oxygen consumption was still elevated (Fig. 1c). The weight loss in this group reached about 7% on the fifth day. The weight remained at this level until about the ninth day, after which it rose toward the preirradiation level (Fig. 2c).

**Sublethal doses.** Doses of 432, 216, 108, and 54 r were administered to 4, 5, 5, and 4 rats respectively. With all 4 doses, the metabolic rate rose slightly (an average of 8%) for a 16-day period following irradiation. The difference is small but highly significant. It

<sup>3</sup> Moses, L. E., *Proc. Soc. Exp. Biol. and Med.*, 1947, **64**, 54.

<sup>4</sup> Fisher, R. A., *Statistical methods for research workers*, 10th ed., 354 pp., N. Y., G. E. Stechert Co., 1946.

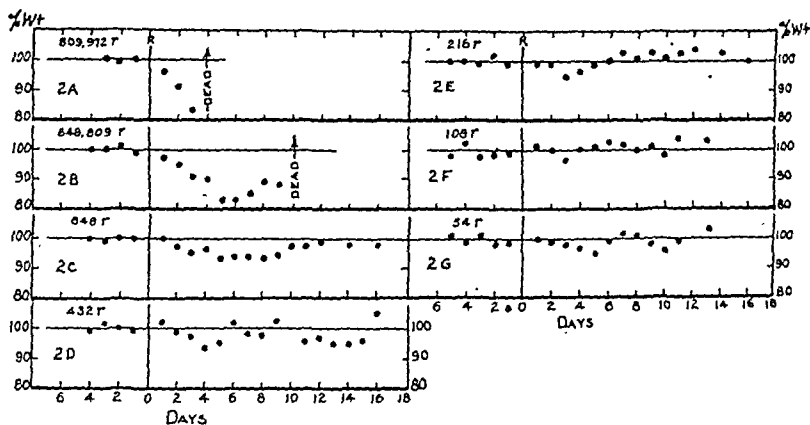


FIG. 2a-g.

Average percentage of body weight before and after irradiation at time R. For each group of rats 100% corresponds to the mean pre-irradiation weight. Doses as indicated.

is based on 74 tests before and 199 tests after irradiation, in a total of 18 rats. The standard deviation of this difference in metabolism between the rats before and after irradiation is about 0.014 or 1.7% of the normal metabolic rate. This is only one-fifth of the difference observed between pre- and postirradiation metabolism. An inspection of the curves (Fig. 1d to 1g) suggests that the rise of the metabolic rate occurs more slowly at these lower doses than in the 3 groups previously described in the lethal range, and that the response does not vary much over the dosage range from 54 to 432 r. Our material is not large enough to warrant any definite assertion beyond the general statement that the metabolic rate does increase after administration of small doses. Weight loss, if it did occur at all, was small and of short duration in the sublethal range (Fig. 2d to 2g).

**Tests with nembutal.** Eight of the experimental rats were lightly anaesthetized with 0.05 g nembutal per 100 g body weight each day before their metabolic rate was measured. The average  $Q_{O_2}$  of the nembutalized animals was 0.79 compared to the control value of 0.82 in 25 unanaesthetized animals tested at about the same time. The response of the metabolic rate to irradiation was about the same in both sets of animals.

**Discussion.** The preceding data show that total body irradiation produces increased oxy-

gen consumption in rats. This reaction occurs over the whole dosage range tested, from 54 to 972 r. The increase is pronounced after lethal doses—whether or not the animal survives—and slight but still significant after sublethal doses. The rise occurs during the first postirradiation day after lethal doses; it seems to develop more slowly after sublethal doses.

The increased oxygen consumption is certainly not due to excessive feeding; irradiated animals eat less than normal. Neither is it due to increased activity, as shown by frequent observations of activity and by the nembutal tests. After high doses, the metabolic rate remains high during a time when the animals are recovering their normal weight, and after small doses the metabolic rate may go up without associated weight loss. Loss of body weight and increase in metabolic rate depend upon consumption of body substance, but there are probably several factors which influence only metabolic rate or body weight and some of these must be responsible for the observed lack of conformity between the two kinds of measurement.

The increase observed in metabolism of the rat after irradiation need not be interpreted as direct stimulation of oxidative enzyme systems. In fact, all available evidence suggests that the metabolic response may result from indirect action. Measurements in buffered saline of respiration of tissue slices from irradiated animals show either a depres-

sion or no change.<sup>5</sup> Similarly respiration is reduced in tissues and isolated enzymes irradiated *in vitro* (Barron *et al.*,<sup>5</sup> Dale<sup>6</sup> and others). Apparently the response of an intact animal where the tissues are bathed by the complex body fluids differs from the response of tissues in simpler media. The body fluids of irradiated animals contain products of tissue breakdown, and there is much evidence of toxemia during the acute radiation reaction. It is not likely that all the toxin is liberated at the time of irradiation. It is more probable that some cell injury remains latent for days and results, over an extensive period, in production of materials which have a specific dynamic action, reflected in elevated metabolism. It is of interest that thyroid metabolism, as indicated by the iodine turnover, is increased 2 to 3 days after irradiation.<sup>7</sup>

<sup>5</sup> Barron, E. S. G., to be published in *Nat. Nucl. En. Ser.*, 1949, IV, 22B.

<sup>6</sup> Dale, W. M., *Brit. J. Rad.*, 1943, 19, 171.

**Summary.** 1. Rats which died 3 to 5 days after total-body irradiation of 809 - 972 r showed an elevation of oxygen consumption of about 35% occurring within 24 hours.

2. Rats which died 9 to 10 days after doses of 648 to 972 r showed an increase in metabolism of about 58% and a decline prior to death.

3. Rats which survived doses of 648 r showed an average increase in oxygen consumption of 32% during the first week, and less pronounced increase extending over a period of at least 16 days after irradiation.

4. At doses from 54 to 432 r there was an increase in metabolism by about 8½%. The maximum oxygen consumption was reached later at sublethal rather than at lethal doses.

5. Increased metabolism did not coincide with weight loss either in time course or in dose dependence.

<sup>7</sup> Evans, T. C., Clarke, G., and Sobel, E., *Anat. Rec.*, 1947, 99, 21.

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## 17226. Differential Blood Counts on Rats During Shock Induced by Tourniquets.

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The hematological changes which occur during secondary shock have been reported by Cannon, Frazer, and Hooper,<sup>1</sup> and other workers as reviewed by Moon.<sup>2</sup> Among the changes reported are variations in the red and white cell counts, but the relative values of the different white cells in particular have been neglected. White and Dougherty<sup>3</sup> have shown that in instances of stimulus or stress there is a dissolution of lymphocytes. It is the purpose of this study to show the changes which occur in the percentages of lymphocytes

and polymorphonuclear neutrophils during induced experimental shock. Through the course of the experiment, it was found that the changes in the percent of monocytes, basophils, and eosinophils were too small to be significant.

**Materials and methods.** Albino rats, Sherman strain, including both sexes, and ranging in weight from 98 to 292 g were used. Rubber bands, tightly wrapped, were placed at the highest possible point on both thighs. The rats were kept in a semi-conscious state with intraperitoneal injections of nembutal (Abbott). Blood was obtained by puncturing a tail vein and then subjected to the usual methods for determining gross and differential counts.

Preliminary experiments on 12 rats were

<sup>1</sup> Cannon, W. B., Frazer, J., and Hooper, A. N., *J.A.M.A.*, 1918, 70, 526.

<sup>2</sup> Moon, V. H., *Shock*, 1942, Lea and Febiger, Philadelphia, Pa.

<sup>3</sup> White, A., and Dougherty, J. F., *Ann. N. Y. Acad. Sci.*, 1946, 46, 859.

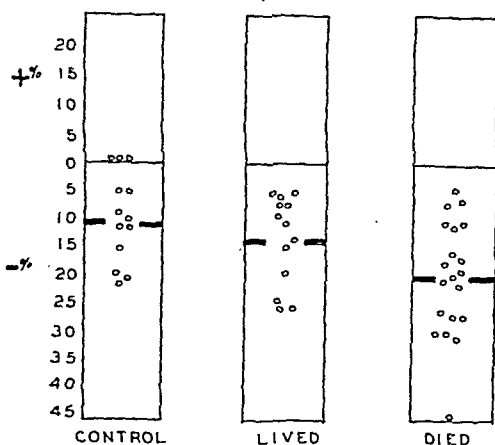
LYMPHOCYTE CHANGES FOR  
CONTROLS AND GROUP 2

FIG. 1.

Each circle represents the change in lymphocytes which occurred between 2 and 3 hours after the release of 5-hour tourniquets in experimental animals and 2 hours after a 5-hour period of anesthesia for the control animals. Broken horizontal bars indicate the average change for each group.

conducted with both gross and differential counts being made at the same time to obtain the actual values of the cells present. The gross counts were discontinued in subsequent experiments after the trend had been established. Another group of 33 rats was used for differential counts alone. To establish the normal count, one smear was made just after the tourniquets were applied, and other smears were made every hour after the removal of the tourniquets for 3 hours if the animal lived. A third group of 11 rats was injected with dibenamine hydrochloride,\* an adrenolytic drug, just before the tourniquets were applied. The procedure, otherwise, was the same as described above. In a fourth group of 9 animals, smears were made from blood taken from the toe at the end of the 5 hour period, just before the removal of the tourniquet. The procedure, otherwise, was the same as described for the second group.

In the present series of shocked rats, an average of 4 counts were made on 144 animals.

\* Supplied by Dr. W. Gump, Giraudan-Delawanna, Inc., Delawanna, N. J.

For each differential count at least 100 cells were counted while in the second group of 33 animals used for differential counts alone and in the control group at least 200 cells were counted. This procedure would appear to establish the validity of the data presented.

**Results.** The significant feature of the studies reported is a comparison between the blood counts of fatal and non-fatal cases of shock. Undoubtedly, the procedures employed, anesthesia, tourniquet trauma, and handling, of themselves will produce some changes in the white blood cell count. However, the decrease in lymphocyte count found in animals that died is significantly greater than variations in the blood count of normal rats. This can be seen in Fig. 1. There are two obvious factors which *per se* may bring about the degree of scatter seen in Fig. 1. First, it should be emphasized that the onset of profound shock varies in the experimental animals and that death occurs anywhere from 1 to 10 hours after release of the tourniquets. Similarly, the greatest lymphocyte decrease is not always 2 to 3 hours after tourniquet release. However, in order to permit a direct comparison between the different animals, the blood samples recorded here were all taken between 2 to 3 hours after tourniquet removal. This factor may be the cause of some of the variability encountered, as illustrated in Fig. 1. Secondly, the animals begin to revive soon after the anesthesia wears off and the stress resulting from continued attempts to escape from the animal-boards may cause a greater temporary decrease in lymphocytes than would be seen if the animals were free. In five rats subjected to pentobarbital anesthesia for a period of 4 hours only a slight decrease in lymphocyte count from 5 to 11%, an average of 9%, was encountered. The average decrease in lymphocytes in controls carried for 2 hours past a 5-hour period of anesthesia (comparable to a 5-hour tourniquet period) was 12.9%. In contrast, the average decrease in fatal cases of shock was 18.5%.

**Group 1. Gross and Differential Counts in Tourniquet Shock.** In preliminary experiments on 12 rats, all of the animals showed a decrease in lymphocytes after removal of the

TABLE I.  
Percentage of Lymphocytes and Time of Death in Dibenzamine Treated Rats 2 Hours after the Release of Hind-Limb Tourniquets.

No.	Normal % of lymphocytes	Lymphocytes 2 hr after tourniquet release, %	Time of death after tourniquet release, hr
1	75	37	3
2	78	61	2
3	66	26	3
4	74	47	3
5	75	57	2
6	64	44	2
7	76	58	2
8	78	49	2
9	66	46	3
10	73	52	2
11	67	46	2

TABLE II.  
Percentage of Lymphocytes in the Leg Prior to Tourniquet Release and 2 Hours after Release.

No.	Normal % of lymphocytes	Lymphocytes in leg before tourniquet release, %	Lymphocytes in blood 2 hr after tourniquet release, %
1	71	75	20
2	65	84	29
3	60	88	54
4	60	56	45
5	94	86	66
6	73	84	53
7	82	92	82
8	79	90	54
9	90	87	50

tourniquets, irrespective of whether the gross count increased or decreased. In all of these cases the *actual* number of lymphocytes was decreased. Six of the rats showed a decrease in gross count following the tourniquet removal. In these the actual number of lymphocytes decreased more than the actual number of polymorphonuclear neutrophils were increased. For example, the normal count of one animal showed a total of 7700 cells, of which 79% (6083) were lymphocytes and 21% (1617) were polymorphonuclear neutrophils. Two hours after tourniquet removal, the gross count was 5800 with 54% (3132 cells) lymphocytes and 46% (2668 cells) neutrophils. Therefore, the decrease in lymphocytes was 2951 cells and the increase in neutrophils was 1051 cells. A similar relationship was found in all instances where there were decreases in gross counts. On the other hand, there was an increase in the gross count in 6 of the rats. In these, the actual increase in the number of neutrophils

was on the average greater than was the decrease in the number of lymphocytes. For example, one animal showed a normal gross count of 6300 cells, of which 90% or 5670 were lymphocytes and 10% or 630 were neutrophils. Two hours after tourniquet removal, the gross count was 6500 cells with 50% or 3250 lymphocytes and 50% or 3250 neutrophils. Therefore, the actual decrease in lymphocytes was 2420 cells while the actual increase in neutrophils was 2620, or 200 cells more.

*Group 2. Differential Counts in Tourniquet Shock.* In a group of 33 rats, 11 died within 4 hours following removal of the tourniquets, 4 died within 6 hours, one died at 21 hours, and 4 died at some undetermined time during the night. Thirteen of the animals lived. The differential counts of the 20 that died showed a decrease in the percent of lymphocytes, the greatest decrease being 46%, the least decrease 4%, and the average 18.8%. Of the 13 animals that lived, the



greatest lymphocyte decrease was 27%, the least decrease 5%, and the average 13.3% (see Fig. 1).

**Group 3. Dibenamine Treated Animals.** In the group of 11 rats injected intraperitoneally with 2 mg dibenamine hydrochloride all of the animals died within 3 hours. These animals showed a considerably shorter period of life following removal of the tourniquet and they also showed a greater lymphocyte decrease than untreated rats (Table I). The greatest lymphocyte decrease was 40%, the least 17%, and the average decrease was 24.4%.

**Group 4. Differential Counts on Blood in Leg Prior to Release of Tourniquets.** In order to determine whether the decrease in lymphocyte count was due to a loss of cells into the traumatized limb, smears were done in 9 rats with blood taken below the tourniquet, just before its removal. These showed an average increase of 11% in lymphocytes. In contrast the decrease in lymphocyte count averaged 24% in the blood taken from the tail vein following the release of the leg tourniquets and the development of shock which resulted in death for all of the animals (Table II).

**Discussion.** Mann<sup>4</sup> has reported a general increase in the number of leucocytes in the initial stages of shock followed by a leucopenia as the animal approaches death. The magnitude of the change which occurred depended on the stage and intensity of the condition.

The group of preliminary experimental rats reported here showed different stages of shock. In the present experiments the initial changes in gross leucocyte counts were inconstant whereas a leucopenia invariably developed within 2 hours, consisting for the most part of a decrease in the percent of lymphocytes. The fact that, as the gross count decreased, the actual number of lymphocytes decreased more than the neutrophils increased, would seem to indicate a definite effect on the individual types of cells. During this general decrease, it would have been less significant,

if the lymphocytes and neutrophils decreased proportionately, maintaining the original ratio.

The lymphocyte decrease would appear to be a result of a reduction in the actual number of cells, caused by decreased production or increased destruction. It has been suggested that lymphocytes may furnish antibodies or inactivate toxins for the body's defense.<sup>5</sup> Dougherty and White<sup>3</sup> have shown that an increase in pituitary-adrenal cortical secretion is paralleled by an increase in lymphocytic dissolution accompanied by an increase in antibody titer in the blood of immunized animals. Selye<sup>6</sup> has also shown that the pituitary-adrenal system is stimulated during the development of shock. The possibility, therefore, exists that this factor may be related to the decrease in the number of lymphocytes.

Munro and Noble<sup>7</sup> found that normal rats, exposed to trauma in the Noble-Collip drum showed a marked fall in lymphocytes. The decrease in number was directly related to the amount of trauma. When the trauma was great enough to cause death the lympholysis was both rapid and severe.

It is possible that the vaso-depressor substance (VDM) described by Shorr, Zweifach and Furchgott<sup>8</sup> as being liberated during the course of shock may be related to the change in lymphocyte count which occurs when the tourniquets have been released.

The early deaths observed in the animals injected with dibenamine hydrochloride may be due to the fact that the compensatory mechanism for combatting the presence of hypotension is made less effective by the action of this drug on the sympathetic nervous system. The secondary changes in the blood picture are similarly enhanced.

**Summary.** 1. A lymphocyte decrease

<sup>5</sup> Best, C. H., and Taylor, N. B., *The Physiological Basis of Medical Practice*, 1945, The Williams and Wilkins Co., Baltimore, Md.

<sup>6</sup> Selye, H., *J. Clin. Endocrinol.*, 1946, **6**, 117.

<sup>7</sup> Munro, D. D., and Noble, R. L., *Fed. Proc.*, 1947, **6**, 168.

<sup>8</sup> Shorr, E., Zweifach, B. W., and Furchgott, R. F., *Science*, 1945, **102**, 489.

<sup>4</sup> Mann, F. C., *Bull. Johns Hopkins Hosp.*, 1914, **25**, 203.

occurs within 2 hours following the removal of the tourniquet in fatal shock induced by leg tourniquets in albino rats. 2. There is no decrease in the percent of lymphocytes in blood smears made below the tourniquet at the end of a 5-hour period. 3. Dibenamine hydrochloride increases the onset of death in

tourniquet-shock and increases the lymphocyte decline.

The writers wish to thank Dr. B. W. Zweifach for helpful suggestions, and also Mr. Jack Chewing and Mr. Joseph Pugh for technical assistance.

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### 17227. Protecting Effect of Heparin on the Inactivation of Thrombin by Heat.\*

L. A. PALOS. (Introduced by J. Tomcsik.)

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Thrombin and thrombokinase, unlike prothrombin, are oxidizable and become inactive in their oxidized form, as reported previously.<sup>1</sup> Further investigations proved that  $\gamma$ -quantities of heparin inhibit completely the inactivation of thrombin by oxidation. This property of heparin, unknown till now, showed itself to be specific; it can be concluded, therefore, that heparin possesses not only an antagonistic action on thrombin, but, under certain circumstances, has also qualities protecting thrombin.<sup>2</sup> The inactivation of the coagulation factors by means of oxidation supports our previous theory on the role of oxidation in blood coagulation. Our experiments show that increased oxidation diminishes coagulability of blood, whereas oxygen deficiency accelerates coagulation. Therefore breathing itself must be the essential regulator in blood coagulation *in vivo* as has been shown.<sup>3</sup> The thrombin-protecting effect of heparin, which contrasts with its inhibitory effect on coagulation, is not clarified in our earlier work. It is supposed that this question could be answered by clearing up the nature of the plasma factor (co-factor) of heparin. This supposition is supported by the fact that the unknown plasma factor can modify the heparin effect, as may be seen by

the different reactions of heparin against plasma and purified fibrinogen. Heparin has no coagulation-inhibitory effect on purified fibrinogen.<sup>4</sup>

In the present work we investigated whether heparin has an inhibiting effect on heat inactivation of thrombin, similar to its protecting effect toward oxidation. We used for our investigations thrombin and heparin (Liquemin, Hoffmann-La Roche). The activity of thrombin was measured with a solution of fibrinogen, containing 15 mg/ml of dry substance; the time of coagulation was noted in seconds. The solutions used for heat inactivation contained thrombin mixed with varied quantities of heparin. In each experiment the mixtures of thrombin and heparin were kept exactly 10 minutes in the water bath. A control series was similarly treated without heparin. The activity of thrombin was measured 1 hour after the samples were removed from the water bath.

It can be seen from Table I that the inactivation of thrombin by heat is inhibited most efficiently by an equal quantity of heparin. Smaller amounts of heparin inhibit less; however, a larger amount does not essentially increase the inhibitory effect. The heat inactivation of thrombin in the presence of an equal amount of heparin is demonstrated in a curve which is essentially similar to the inactivation curve of thrombin following oxidation.

\* Aided by the Roche Research Fund, Basel.

1 Palos, L. A., *Nature*, in print.

2 Palos, L. A., *Experientia*, 1949, 5, 207.

3 Palos, L. A., *Acta Medica Scandinavica*, in print.

4 Howell, W. H., and Holt, E., *Am. J. Physiol.*, 1918-19, 47, 328.

TABLE I.  
Activity of Thrombin after Keeping 10 Min. at Different Temperatures in Water Bath with and without Heparin.

Temp. °C	Thrombin:	1 mg	1 mg	1 mg	1 mg
	Heparin: pro ml dest water	1.5 mg	1 mg	0.5 mg	0 mg
20		15"	15"	16"	16"
30		16"	16"	20"	20"
40		17"	18"	22"	29"
50		23"	24"	34"	50"
60		25"	26"	51"	500"
70		28"	29"	66"	1200"
80		34"	36"	88"	∞
90		44"	45"	90"	∞
100		51"	52"	260"	∞

Without heparin inactivation of thrombin is strongly accelerated at 50°C; between 70 and 80°C it loses its activity, *i.e.*, the fibrinogen cannot be further coagulated. In contrast, a solution of thrombin containing a corresponding amount of heparin, after even 10 min. boiling (100°C), causes a strong coagulation of fibrinogen; the activity diminishes only to one-third of the initial value.

The protecting effect of heparin shows differences concerning oxidation of thrombin and inactivation by heat. In the first case  $\gamma$ -quan-

ties of heparin were enough to stop oxidation completely. For protecting the inactivation by heat there are required at least equal quantities of heparin to produce a marked effect. Total inhibition of heat inactivation could not be obtained. If the treatment with heat is prolonged, the thrombin loses its activity despite the protecting effect of heparin, but much more slowly than without heparin.

A similar effect to that of heparin could not be produced with other, not esterified di- and polysaccharides, neither in the oxidation experiments nor in the investigation on heat inactivation.

Synthetic polysaccharidesulfonates, which correspond to heparin in inhibition of coagulation, were not investigated.

These new facts, the total inhibition of oxidation and the high grade of inhibition of inactivation of the thrombin by heparin may lead to a new conception of the relationship between thrombin and heparin.

**Summary.** The inactivation of thrombin by heat is sharply inhibited, if it is mixed with equal parts of heparin. Heating a mixture of thrombin-heparin (1:1) for 10 minutes to 100°C causes only a slight decrease of activity.

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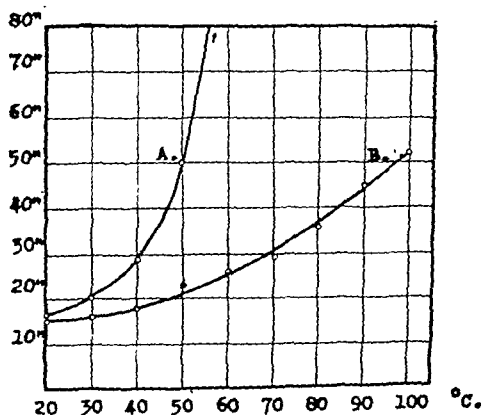


FIG. 1.

Activity of thrombin after keeping 10 minutes at different temperatures in water bath without (A) and with (B) heparin.

# 17228. Relationship Between Spontaneous Activity and Metabolic Rate as Influenced by Certain Sympathomimetic Compounds.

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The measurement of spontaneous activity in small animals appears to be dependent upon the method used, many of which have been described in the literature. Results reported by the author,<sup>1</sup> based upon change of resistance in an electrical circuit when an electrode is moved in and out of a salt solution, differ from those obtained by others. Schulte *et al.*,<sup>2</sup> using a work adder type of kinisimeter, reported that d-N-methyl- $\alpha$ -methyl- $\beta$ -phenylethylamine HCl (desoxyephedrine) was a more powerful stimulant than dl-benzedrine HCl. They also found that the l-isomer of N-methyl- $\beta$ -cyclohexylisopropylamine HCl had a greater stimulating effect than the d-isomer. The writer was unable to confirm either of these results. The results obtained with all kinisimeters may be criticized on the basis that they do not record the very small movements pro-

duced by compounds, such as desoxyephedrine which causes the animal to swing his head continuously in a rotary manner. Since oxygen is consumed in making these movements, it was considered that a measurement of oxygen consumption and determination of the metabolic rate would be of use in determining spontaneous activity effects.

**Apparatus.** The types of apparatus used for oxygen consumption determination have been reviewed by Tabor and Rosenthal.<sup>3</sup> The apparatus used in this study is shown in Fig. 1. The tops of two 500 ml dispensing burettes were connected to a large pyrex desiccator. A thermometer was fastened to the inside wall of the desiccator in order that the vapor tension could be corrected. The upper ends of the burettes were connected to specially constructed manometers filled with a saturated solution of sodium chloride containing a small amount of aerosol to decrease surface tension. Two expansion chambers are included in each of the manometers to prevent the electrolyte from being accidentally drawn into the burettes or spilled to the outside when the apparatus is being filled with oxygen. It will be noted that a small vent is blown in the side wall of the expansion chamber containing the electrodes. These electrodes control the current passing through Allen and Bradley solenoids, which, in turn, control the flow of water from the 500 ml leveling bulbs into the burettes. A simple switch is included in each solenoid circuit in order that the current may be broken at will, to permit filling the burettes with oxygen. The base of the desiccator is filled with soda lime and a disc of heavy screen wire (hardware cloth) replaces the desiccator plate.

All measurements are made in a constant temperature room. The animal is placed in the chamber and the apparatus washed out

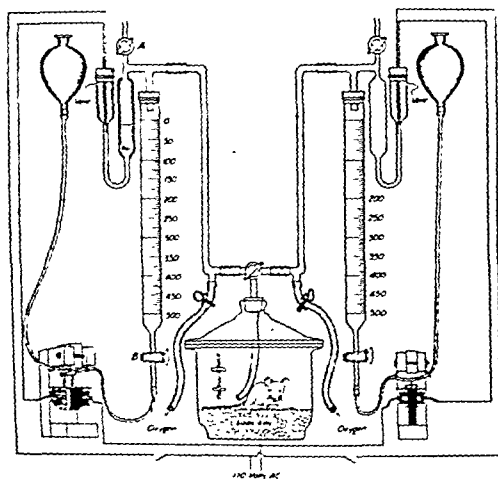


FIG. 1.

Apparatus for the continuous estimation of the metabolic rate of small animals.

<sup>1</sup> Waterman, F. A., *Science*, 1947, 106, 499.

<sup>2</sup> Schulte, J. W., Reig, E. C., Bacher, J. A., Jr., Lawrence, W. C., and Tainter, M. L., *J. Pharm. Exp. Therap.*, 1941, 71, 62.

<sup>3</sup> Tabor and Rosenthal, *Am. J. Physiol.*, 1947, 149, 449.

several times with oxygen. As the animal consumes oxygen the side arm of the manometer falls and breaks the electrical circuit, causing the solenoid to drop and allowing water to enter the burette. When the water has replaced the consumed oxygen, the original level of the electrolyte in the manometer is restored, automatically closing the circuit and the solenoid again pinches the rubber tube which stops the flow of water. When one burette is depleted of oxygen, the 3-way stopcock is turned and the second burette is thrown into service while the first one is being refilled. Thus the oxygen consumed can be determined continuously.

In the calculations an R.Q. of 0.725 was assumed.<sup>4</sup> The metabolic rate in this investigation is defined as calories of heat per hour per gram of body weight. All measurements were made with animal in the post absorptive state. This method is particularly useful for the rapid screening of drugs. The accuracy of such methods as that of Haldane<sup>6</sup> and ac-

curate surface area determinations are unnecessary.

**Experimental.** Control animals were injected with normal saline solution and their metabolic rate determined at 10-minute intervals over a period of 6 hours. The experimental animals were injected with the compound being studied and determinations of oxygen consumption in 10-minute intervals were begun at once. The control and experimental data obtained were plotted along with the data previously obtained from activity studies<sup>1</sup> in order that the relationship between metabolic rate and activity could be readily seen. The following compounds were studied:

	No. of rats	Dosage (mg/kg)
d-N-methyl- $\alpha$ -methyl- $\beta$ -phenylethylamine HCl (desoxyephedrine)	6	5
dl-benzedrine HCl	6	5
1(3',4'-dihydroxyphenyl)-2-isopropylaminoethanol HCl (Isuprel)	6	1
N-methyl- $\beta$ -cyclohexylisopropylamine HCl		
d-isomer	6	5
l-isomer	6	5
Neosynephrine HCl	6	0.07

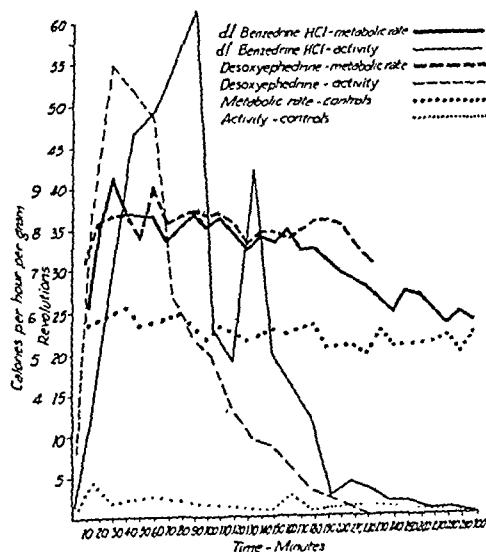


FIG. 2.

Graph showing the effect of dl-benzedrine and desoxyephedrine on the metabolic rate and central nervous excitation.

<sup>4</sup> Griffith and Farris, 1942, *The Rat in Laboratory Investigation*, p. 186.

<sup>5</sup> Diack, Samuel L., *J. Nutrition*, 1930, **3**, 289.

<sup>6</sup> Haldane, J. B. S., *J. Physiol.*, 1892, **13**, 419.

Fig. 2 shows the effects of both benzedrine and desoxyephedrine (d-N-methyl- $\alpha$ -methyl- $\beta$ -phenylethylamine HCl) on the metabolic rate and activity of the animals. The maximum activity as well as the maximum metabolic rate are higher in the case of benzedrine

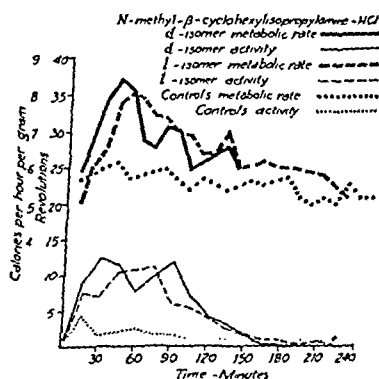


FIG. 3.

The relationship between the d- and l-isomers of N-methyl- $\beta$ -cyclohexylisopropylamine HCl as regards their effect on metabolic rate and central nervous excitation.

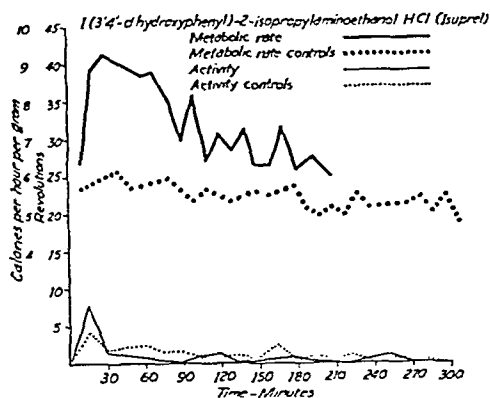


FIG. 4.

The effect of Isuprel on the metabolic rate and central nervous excitation.

than in the case of desoxyephedrine.

The effects produced by the d- and l-isomers of N-methyl- $\beta$ -cyclohexylisopropylamine HCl are shown in Fig. 3. The d-isomer is more active than the l-isomer as shown by effects on both activity and the metabolic rate. All of the above-mentioned compounds show parallel increases in both activity and metabolic rate.

The injection of 1(3',4'-dihydroxyphenyl)-2-isopropylaminoethanol HCl (Isuprel), Fig. 4, produces, if anything, a slight decrease in the activity of the normal animal. However, the metabolic rate is markedly increased, being even higher than that produced by dl-benzedrine. The animal is in a state of com-

plete collapse and breathing rapidly. His heart rate is greatly increased, the peripheral vessels are greatly dilated and the temperature is elevated. The effect on the metabolic rate is clearly not due to movements produced by central nervous stimulation. This compound has been shown to have a relaxing effect on bronchiole spasm induced by pilocarpine and to markedly decrease the blood pressure.<sup>7</sup> The increased oxygen consumption is attributable in part to increased heart rate, respiratory movements, and possibly to increased tissue oxidation.

**Summary.** Comparisons were made between the oxygen consumption and increased movements induced by the central nervous excitatory effects of a number of physiologically active organic compounds. After injection of several of these compounds, benzedrine, desoxyephedrine and N-methyl- $\beta$ -cyclohexylisopropylamine HCl, oxygen consumption and voluntary movements varied in direct relationship to each other. After the injection of another compound (Isuprel), oxygen consumption increased greatly, but there was a decrease in activity. Oxygen consumption can be used as a measure of voluntary muscular activity only under limited conditions.

<sup>7</sup> Lands, A. M., personal communication.

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## 17229. A Simplified Tissue Culture Technic.

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In a single chicken embryo, we find all that is required for preparing tissue cultures: the ingredients of the culture medium as well as the explant. Salt-glucose mixtures are replaced by amniotic fluid from the same embryo from which the tissues are obtained. An egg shell sector about 1¼ inches in diameter is cut out of the large egg pole on the 8-9th day of incubation, and the contents are

poured cautiously into a Petri dish so as to avoid bursting the very delicate amniotic membrane. The membrane is then punched with a thin, sharp cannula and 2-3 cc of amniotic fluid are aspirated. The shell sector should not be larger than indicated, otherwise the amniotic membrane may be easily damaged. Only completely transparent fluid should be used; turbid fluids are mixtures of yolk and amniotic fluid and will inhibit growth. Tissue for explants is then cut out

\* Fellow of the World's Health Organization.



FIG. 1.

Photomicrograph of a living culture of chick embryo heart tissue cultured in embryonic juice and + amniotic fluid (1:1), all obtained from the same embryo.

and the remaining embryonic tissue provides the embryonic juice. The culture medium consists of embryonic juice and amniotic fluid 1:1. The excess of the amniotic fluid may be used for all purposes where saline or other salt mixtures are usually used, e.g., washing of tissue, etc. The growth of cultures in this medium is not inferior to the growth of cultures in other fluid media. The cells are as normal in appearance as cells from a plasma culture (Fig. 1). Amniotic fluid not only replaces but also, in many regards, surpasses Tyrode's and other salt-glucose solutions in that it does not require sterilizing, filtering, or pH-adjusting. This liquid medium method may be recommended for cultures where the primary medium is to be removed for experimental purposes and replaced by other media. It is also convenient for obtaining growth of single cell layers for investigation in dark-field illumination, in phase contrast microscopy and in electron microscopy.

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### 17230. A Method of Obtaining Influenza Virus Growth Curves in Individual Eggs.\*

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Growth curves of influenza virus propagated in the allantoic sac of embryonated eggs have been described by several investigators.<sup>1-6</sup>

\* Aided by a grant from the United States Public Health Service.

† James Hudson Brown Memorial Research Fellow.

<sup>1</sup> Miller, G. L., *J. Exp. Med.*, 1944, **79**, 173.

<sup>2</sup> Henle, W., and Henle, G., *Am. J. Med. Sci.* 1944, **207**, 705.

<sup>3</sup> Ziegler, J. E., Jr., and Horsfall, F. L., Jr., *J. Exp. Med.*, 1944, **79**, 361.

<sup>4</sup> Henle, W., Henle, G., and Rosenberg, E. B., *J. Exp. Med.*, 1947, **86**, 423.

<sup>5</sup> Hoyle, L., *Brit. J. Exp. Path.*, 1948, **29**, 390.

<sup>6</sup> Henle, W., and Rosenberg, E. B., *J. Exp. Med.*, 1949, **89**, 279.

However, most of the studies reported have been based on results obtained with the pooled allantoic fluids of groups of eggs sacrificed at various intervals of time following inoculation of virus. Relatively little data concerning the characteristics of such curves in individual eggs are available. The use of pooled fluids has yielded valuable information as to the average rate and extent of virus multiplication and, with the aid of special technics, the step-like nature of the curves.<sup>4-6</sup> Growth curves in individual eggs should provide similar information, in addition to presenting a continuous picture of the growth curve in each egg. Recently Hoyle<sup>5</sup> has described growth curves of influenza A virus

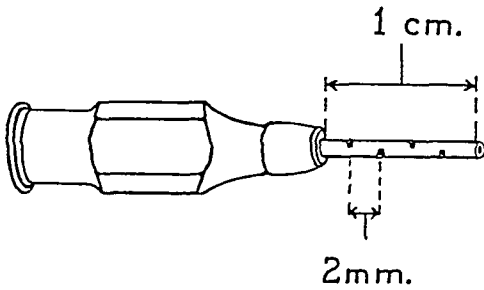


FIG. 1.

Needle devised for aspirating allantoic fluid.

in individual eggs from which allantoic fluid was removed at hourly intervals. However, no details of the technic used for sampling were given by him. The present paper describes a method by which individual growth curves may be obtained.

**Materials and methods.** Samples of allantoic fluid were aspirated through needles especially devised for this purpose. Aspirating needles were made from ordinary 20 gauge "hypodermic" needles by cutting off the ends and filing 4 holes in the sides of the short lengths of needle remaining. As illustrated in Fig. 1, the length of the needle, from attachment to the hub to the tip, is one centimeter. The holes along the shaft are spaced alternately on opposite sides, 2 mm apart, the first hole being 2 mm from the tip and the fourth 2 mm from the hub. A needle of this type was made readily, in 5 to 10 minutes, with the aid of the flat metal file commonly used to remove the tips of glass ampoules. However, the filing could be done more efficiently by means of a dental drill fitted with a flat cutting disc. During the process of filing, the needle was held firmly by attaching it to a small syringe equipped with a "Luer-Lok" device, and a stylet was inserted before making the side holes.

Needles somewhat similar to those described above have been used previously, especially for flushing the allantoic sac,<sup>7</sup> and for collecting large amounts of allantoic fluid.<sup>8</sup>

Preparatory to inoculation of virus, each egg was candled and a mark was made on the side of the shell in the vicinity of the embryo, directly over an area of chorio-allantoic membrane where no large blood vessels were seen. A hole of about the same size as a cross section of the needle was then drilled through the shell at the designated mark. The hole used for inoculation of virus, as a rule, was used for subsequent aspirations. After inoculation and after each aspiration the holes were sealed with a drop of paraffin-bee's wax mixture. Prior to aspiration the eggs were candled to determine viability; the wax seals were removed with a hot metal spatula and the areas about the holes cleansed with alcohol. After attachment to a syringe of suitable capacity the needle was slowly inserted to the hilt and suction applied by withdrawing the plunger. At each aspiration exactly 0.2 cc of allantoic fluid was withdrawn and added to 0.8 cc of 0.85% NaCl. Serial two-fold dilutions were then made in saline in 0.5 cc amounts. To each tube 0.25 cc of 1% chicken RBC was added and readings were made after the cells had settled out, on standing at room temperature for about an hour. The end-point was taken as the highest dilution of allantoic fluid in which a pattern characteristic of complete or ++++ agglutination occurred. The PR8 strain of influenza A virus was used exclusively.

**Experimental.** Groups of 10 to 20 embryonated hens' eggs, on the tenth day of incubation, were inoculated with influenza virus via the allantoic sac. As a rule, the inoculum consisted of approximately 100 ID<sub>50</sub> in a volume of 0.05 cc. The eggs were subsequently incubated at 37°C. Chilling was not done, and the eggs were removed from the incubator only for the brief periods of time required for each aspiration. When small inocula of virus were used, the first aspiration was done at 18 hours after inoculation because preliminary experiments had shown that hemagglutinins usually could not be demonstrated before that time. Repeated aspirations were then done at arbitrarily chosen intervals as long as the embryos survived.

In Fig. 2 are shown the curves, representing

<sup>7</sup> Henle, W., and Henle, G., *Am. J. Med. Sci.*, 1944, **207**, 717.

<sup>8</sup> Taylor, A. R., Sharp, D. G., McLean, I. W., Jr., Beard, D., and Beard, J. W., *J. Immunol.*, 1945, **50**, 291.



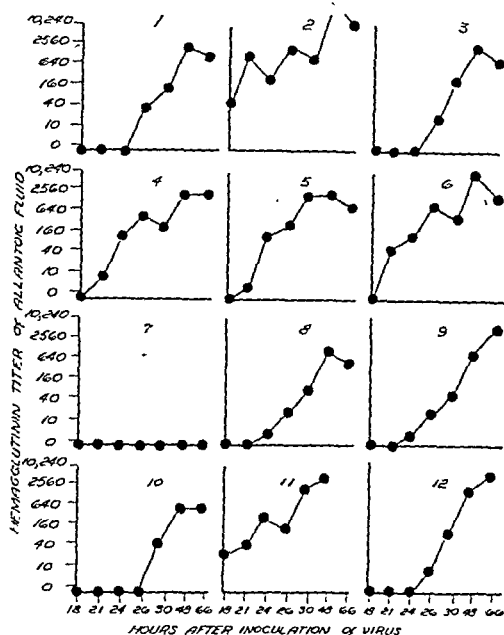


FIG. 2.

Growth curves, based on hemagglutination titers, of the PR 8 strain of influenza virus in individual eggs. Results are those obtained in a single experiment, in which each of 12 eggs received an inoculum of approximately 100 ID<sub>50</sub> of virus.

the serial hemagglutination titers in individual eggs, obtained in an experiment in which each of a group of 12 eggs received a standard inoculum of about 100 ID<sub>50</sub> of virus. While some of the curves are quite similar, others show obvious differences with respect to time of appearance of detectable hemagglutinin, slope of curve and maximum titer of hemagglutinin. Moreover, as previously demonstrated by Henle<sup>4,6</sup> and Hoyle,<sup>5</sup> several of the eggs, especially numbers 2, 4, 6 and 11, show step-like increases in the amount of virus. The fact that egg number 7 never became positive may have been due to failure to inoculate

it; however, in other similar experiments, an occasional egg has shown an even longer lag phase, hemagglutinins first making their appearance some 90 hours or more after inoculation. All embryos in this experiment were alive 48 hours after inoculation of virus but when candled just prior to the 66 hour aspiration all except number 10 were found to be dead. In most similar experiments a rather high mortality has occurred somewhat earlier.

*Comment.* The method described is technically easy; relatively small numbers of eggs are required, and the growth curves obtained are individual curves rather than those based on group sampling. Furthermore, this method provides a suitable way of studying the differences between curves in untreated eggs and those in eggs treated with various substances which inhibit the multiplication of influenza virus. However, a high mortality rate usually occurs and about 50% of the eggs die before satisfactory growth curves are obtained. Mortality varies considerably from one experiment to another and apparently depends upon a number of factors which have not been investigated fully. Only a small percentage of the eggs become contaminated with bacteria. In most instances aspiration yields clear allantoic fluid, but occasionally bloody fluid or yolk is obtained. A higher mortality and greater difficulty in aspiration are most likely to be encountered with eggs which have been incubated for only 10 days or less.

*Summary.* With the aid of an easily constructed needle a method has been devised for obtaining growth curves of influenza virus in individual embryonated eggs.

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# 17231. Some Effects of Dilution on the Nutritive Value of Dialyzed Plasma and Embryo Juice.\*

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The classic substratum for tissue cultures, developed by Harrison,<sup>1</sup> Burrows,<sup>2</sup> and Carrel,<sup>3</sup> consists of chick plasma clotted with chick embryo juice and with an overlay of embryo juice variously diluted. Such a plasma clot without embryo juice overlay is insufficient to support prolonged tissue growth except when frequently renewed by transplanting the cultures to fresh substratum. It supplies an ill-defined but far from negligible level of nutritional factors. In any precise study of cell nutrition, these factors must either be clearly defined or, if possible, eliminated before the substratum can be satisfactorily evaluated as a base line. Various attempts have been made to arrive at such an evaluable base line. Porter and Hawn<sup>4</sup> have used clots prepared from purified fibrinogen coagulated with thrombin. Both these substances are more clearly defined and more nearly inert than are plasma and embryo juice, but they are still not completely defined. Evans and Earle<sup>5</sup> have been successful in developing a procedure for cultivation of tissue cultures on sheets of perforated cellophane, which is nutritionally fully inert. This is a very satisfactory solution of the nutritional problem, but such cellophane is not easily available to all workers throughout the world. Lewis,<sup>6</sup> White,<sup>7</sup> and others have

grown cultures directly on glass. This is likewise a fully satisfactory solution of the nutritional problem but the results obtained are somewhat unreliable.

Fischer<sup>8</sup> has approached the problem from quite a different angle by seeking to eliminate the nutritional factors from the classic plasma clot. He showed that when chick plasma and embryo juice, after thorough dialysis against a dextrose-Ringer's solution, were used in the preparation of clots on which to cultivate chick fibroblasts, no growth whatever would occur thereon. He concluded that such a clot was nutritionally inert and was not attacked by the tissue enzymes. This being true, it could safely be used as an inert substratum against which to study the nutritive properties of various supernatants. Dialyzed plasma and dialyzed embryo juice have been rather extensively used in such studies.<sup>9</sup>

Fischer states<sup>8</sup> that implanted tissues grew less and disintegrated more quickly on dialyzed plasma than they did in Tyrode's solution alone. If the defect is merely one of nutrient deficiency, it is difficult to see how Tyrode's solution could be the less deficient of the two. The alternative possibility exists that the defect may be due not merely to a low level of nutritive value but to some more positive injurious effect. Indeed, Fischer himself speaks of "den Eindruck einer direkten toxischen Einwirkung."<sup>8</sup> For this and other reasons we decided to repeat Fischer's work. Preliminary experiments were carried out by somewhat simplified procedures, using hanging drop cultures in place of Carrel flasks and chick heart fibroblasts in place of frontal bone fibroblasts.

Fischer used an undiluted dialyzed embryo

\* Aided by a grant from the American Cancer Society, recommended by the Committee on Growth of the National Research Council.

<sup>1</sup> Harrison, R. G., *Proc. Soc. Exp. Biol. and Med.*, 1907, **4**, 140.

<sup>2</sup> Burrows, M. T., *J. Am. Med. Assn.*, 1910, **55**, 2057.

<sup>3</sup> Carrel, A., *J. Exp. Med.*, 1913, **17**, 14.

<sup>4</sup> Porter, K. R., and Hawn, C. v. Z., *Proc. Soc. Exp. Biol. and Med.*, 1947, **65**, 309.

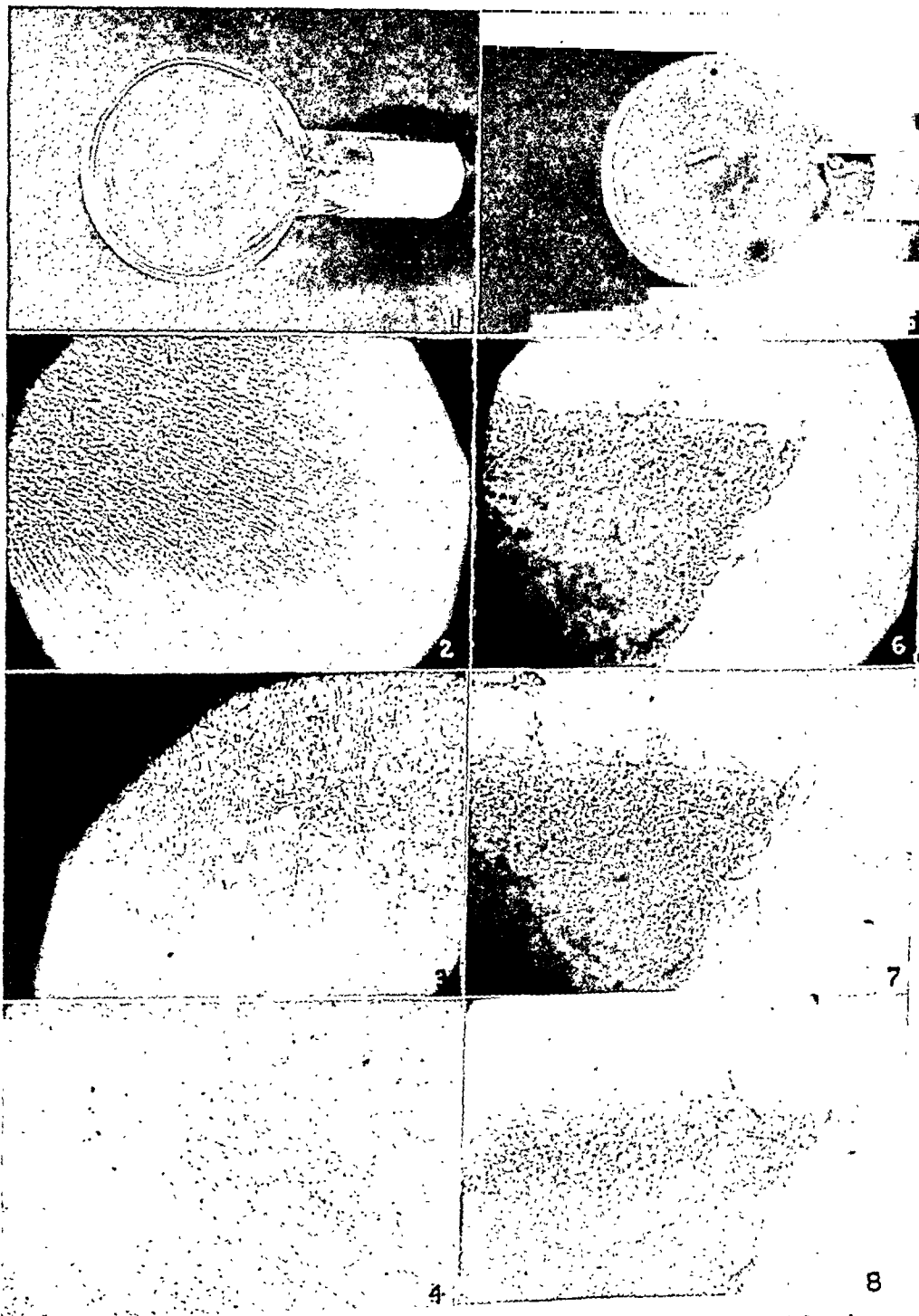
<sup>5</sup> Evans, V. J., and Earle, W. R., *J. Nat. Cancer Inst.*, 1947, **8**, 103.

<sup>6</sup> Lewis, Warren H., *Carnegie Inst. Wash., Contrib. to Embryology*, 1926, **18**, 1.

<sup>7</sup> White, P. R., *Growth*, 1947, **10**, 231.

<sup>8</sup> Fischer, A., *Acta Physiologica Scandinavica*, 1941, **2**, 143.

<sup>9</sup> Fischer, A., Astrup, T., Ehrensward, G., and Oelenschlager, V., *Proc. Soc. Exp. Biol. and Med.*, 1948, **67**, 40.



(All photographs are of living material, made with 16 mm panatomic film. Fig. 1, 5, 9, and 13 (Carrel flasks) are  $\times 1$ . All others are about  $\times 76$ ).

FIG. 1. Twenty-four hour culture of chick frontal bone fibroblasts on the control medium consisting of chick plasma clot with overlay of chick embryo juice diluted 1:9 with Tyrode's solution. The halo of migrating cells is clearly evident.

FIG. 2. Detail from the above culture showing the compact, uniform growth of finely granular cells.

FIG. 3. Margin of the same culture at the end of 4 days. The cells contain many small fat globules and are larger than in Fig. 2, but are in excellent condition.

FIG. 4. Area from about midway between the original implant and the edge of the flask, taken at the end of 10 days. The uniform character of the growth and relatively clear cytoplasm of the cells are evident.

FIG. 5. Twenty-four hour culture of chick frontal bone fibroblasts on an undiluted dialyzed plasma-dialyzed embryo juice clot, with undiluted dialyzed embryo juice overlay. No growth has occurred.

FIG. 6, 7, 8. Details of the above culture after 24 hours, 4 days, and 10 days. There has been no migration or change other than disintegration of the cells within the implanted bit of clot.

juice in the preparation of both his substratum and his experimental supernatant.<sup>8</sup> We have obtained our most satisfactory control results (undialyzed media) with nutrients in which the embryo juice\* was diluted about 1 to 9 with Tyrode's solution. In order that our experimental cultures might be more nearly comparable with the controls, we therefore carried out cultures not only with undiluted embryo juice but with dilutions approximating those of our controls. Our experiments with undiluted embryo juice fully confirm Fischer's findings, but, to our surprise, cultures prepared with diluted dialyzed embryo juice grew quite well over the 48-hour periods which can be followed in hanging drops. This suggested that the undiluted material remaining after dialysis was perhaps not *deficient*, that is, lacking in essential nutrient factors, but merely *defective* due to unsatisfactory concentrations or proportions of its constituents. These initial experiments were, however, not duplicates of those reported by Fischer. We therefore carried out another series of cultures, repeating as nearly as possible Fischer's second procedure<sup>8</sup> and extending the study to the use of diluted media.

One of us (E.L.) spent a month in the summer of 1946 in Fischer's laboratory in Copenhagen and was therefore familiar with his methods. In the preparation of plasma and in dialysis of both plasma and embryo juice we followed Fischer's procedures ex-

actly. Twenty-five ml of plasma, from blood drawn by heart puncture from a young cockerel into a heparinized syringe, were transferred to a sterile (autoclaved) cellophane dialysis tube under sterile precautions and were dialyzed for 8 days in the refrigerator at about 4°C against 1.5 liters of Ringer's solution containing 0.1% dextrose. The solution was not renewed.<sup>8</sup> Freshly prepared juice from 9 day chick embryos was similarly treated. All dilutions were made with Tyrode's solution without dextrose. It was found that satisfactory clots could not be obtained with dilutions higher than one part of embryo juice in two parts of Tyrode's solution. A series was set up with 16 Carrel flasks, 4 flasks containing each of the following solutions: 1) control, undiluted plasma (0.5 ml) coagulated with 0.5 ml of embryo juice diluted 1:2, supplied after clotting with a supernatant of embryo juice diluted 1:9; 2) Fischer's cultures, consisting of undiluted dialyzed plasma (0.5 ml) clotted with 0.5 ml of undiluted dialyzed embryo juice, and with a supernatant of undiluted dialyzed embryo juice; 3) similar to the latter, but with 0.5 ml of undiluted dialyzed plasma clotted with dialyzed embryo juice diluted with an equal part of Tyrode's solution (0.5 ml), and a supernatant of dialyzed embryo juice likewise diluted with an equal part of Tyrode's solution; and 4) undiluted dialyzed plasma (0.5 ml) clotted with dialyzed embryo juice diluted with 2 parts of Tyrode's solution (0.5 ml), and a supernatant of dialyzed embryo juice similarly diluted. In each flask was placed a single fragment of a rapidly growing homogeneous culture of fibroblasts derived from the frontal bone of a 14-day chick em-

\* Embryo juice was prepared by finely triturating with sharp scissors without addition of fluid, allowing to stand for one-half hour, and then centrifuging.

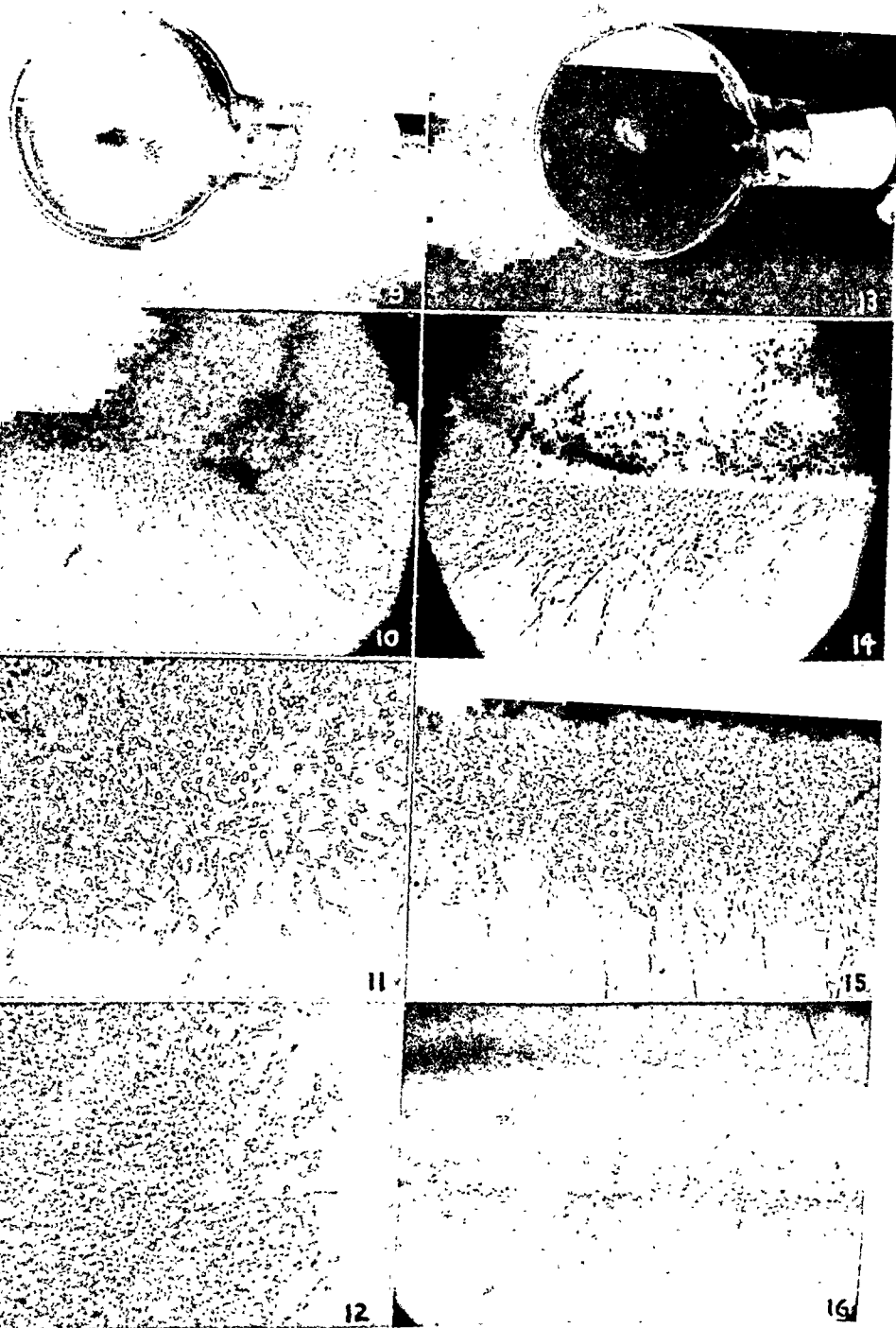


FIG. 9. Twenty-four hour culture of chick frontal bone fibroblasts on a clot prepared with dialyzed embryo juice diluted 1:1 with a dextrose-free Tyrode's solution, with overlay of this

same dilution. The halo of migrating cells is much smaller than in Fig. 1, but is still quite evident.

Fig. 10. Detail from the above culture showing the sparse but evident migration of fibroblasts.

Fig. 11. The same culture as Fig. 10 at the end of 4 days (compare with Fig. 3). The zone of migrating cells is fairly wide and quite uniform. The cells themselves are large, larger than in Fig. 3, but are packed with fat globules, many of which are very large.

Fig. 12. The same culture after 10 days. Outlines of single cells are no longer visible, disintegration having taken place.

Fig. 13. Twenty-four hour culture of chick frontal bone fibroblasts on a clot prepared with dialyzed embryo juice diluted 1:2 with dextrose-free Tyrode's solution, with overlay of the same dilution. The considerable halo of migrating fibroblasts is evident.

Fig. 14. Detail from the above culture. The area of growth is both qualitatively and quantitatively better than at a 1:1 dilution (Fig. 10).

Fig. 15. The same culture after 4 days. Growth is somewhat more compact than at a 1:1 dilution (Fig. 11) but otherwise not very different.

Fig. 16. The same after 10 days. Cells in the original migrating zone (transverse band) are fatty but in the gap left between this and the original implant by lysis and contraction of the clot, subsequently patched, the new cells have clear-cut outlines and are not excessively filled with fat. This culture is still very much alive and growing, though very slowly.

bryo and maintained on the standard chick plasma-dilute embryo juice medium through four successive passages. Cultures were examined and photographed at the end of 48 hours, 4 days, and 10 days. Nutrient was replaced in each of the flasks after 2, 4, 6 and 8 days.

**Results.** The results were clear cut, consistent both among themselves and with the results of previous experiments using hanging drop cultures. Growth on the control nutrient was normal and very rapid, so that at the end of 10 days the entire *bottoms* of the flasks were covered. The clot had undergone lysis around the implant and was patched on the 4th day; new growth had covered the patch by the 10th day (Fig. 1-4). On the undiluted dialyzed plasma dialyzed embryo juice medium, in complete agreement with Fischer's results, there had been absolutely no growth and the cells had undergone histolysis (Fig. 5-8). On dialyzed plasma containing dialyzed embryo juice diluted 1:1, however, there had been very considerable growth (migration). At 24 hours this reached a maximum width of about 0.1 mm. At 4 days the band was about 0.8 mm wide, the cells were *larger* than the controls and were packed with fat globules. At 10 days they had grown no further and were mostly dead and broken down (Fig. 9-12). Dialyzed plasma clotted with embryo juice diluted 1:2 did not give firm uniform clots, although they were sufficiently coherent to attach the cultures satisfactorily and to provide a satisfactory sub-

stratum on which growth could take place. At 24 hours the growth zone was about 0.5 mm wide, 5 times as great as at 1:1 dilution. At 4 days this had about doubled, that is, was about the same width as in the 1:1 dilution. The growth was, however, denser and the cells somewhat less fatty. At 10 days, some lysis had occurred (patched at 8 days), the band was about 2 mm wide, cells had grown out into the patched areas, and the cells were still alive (Fig. 13-16).

**Discussion.** The results agree with those of Fischer<sup>8</sup> in showing that an undiluted dialyzed plasma-dialyzed embryo juice clot with undiluted dialyzed embryo juice overlay does not support growth of a homogeneous strain of frontal bone fibroblasts and brings about rapid breakdown of implanted cells. Yet dilution of the overlay with Tyrode's solution lacking dextrose, which is a non-nutritive and purely protective solution, restores the capacity to support at least a residual degree of growth, and the degree of restoration is, within the limits set by the clotting capacity of the medium, proportionate to the degree of dilution. It is evident that the failure of the undiluted dialyzed material to support growth was not due solely to a deficiency, since any deficiency would only have been accentuated by dilution. It must, therefore, have been due to some positive defect. Such positive defects may be of two sorts. There may be present a non-dialyzable growth inhibitor, either a high molecular weight substance or a prosthetic

group attached to such a substance, whose effects are minimized by dilution. One point in Fischer's data might lend support to the idea of such a toxic factor. Fischer reports that either egg albumin or insulin, when added to dialyzed plasma, will protect cells placed thereon from histolytic disintegration. He attributes this effect to the nutritive properties of the labile S-S and S-H bonds in these materials, but both egg albumin and insulin are known to be highly active adsorbents capable of detoxifying various media. This appears to be the effect of serum albumin when added to media for the cultivation of bacteria<sup>10</sup> and may have been a factor in Spratt's experiments<sup>11</sup> with explanted early chick blastoderms (not tissue cultures). It seems quite possible that the beneficial effect of egg albumin or insulin when added to dialyzed plasma may be of similar origin. On the other hand, the defect might be due to an imbalance of nutritive materials. The plasma and embryo juice were dialyzed against a Ringer's solution containing 0.1% dextrose but lacking phosphate. The diluent used in preparing media (Tyrode's solution) contained no dextrose but did contain phosphate. The diluent was thus *not* in equilibrium with the dialyzed materials, and would both extract dextrose therefrom and

add phosphate thereto. If 0.1% dextrose was too high a concentration for fibroblast growth, the defect would be corrected by dilution. Since phosphate is required for growth, the defect might be a simple deficiency as postulated by Fischer, but not necessarily a deficiency of organic materials. However, Fischer's evidence, that a suitable amino-acid mixture prepared with physiological salt solution rather than Tyrode's solution does, in fact, partially correct the defect of dialyzed plasma-embryo juice, shows that phosphate was not the critical factor. It shows also that the low molecular weight materials removed by dialysis, such as the amino acids, are of great and at this level critical importance.

All of this evidence, however, still leaves unresolved the question of the nature of the postulated positive toxic defect in the dialyzed materials. In any case, our observations, though limited, suggest that it is unsafe to assume that dialyzed plasma or embryo juice is truly inert. Until further experiments have been carried out to clarify the effects of dilution on dialyzed extract, we feel that these materials should not be used in the preparation of substrata on which to test the nutritive value of supernatants. We believe that bare glass or cellophane are still the only really safe bases for such studies.

<sup>10</sup> Davis, B. D., and Dubos, R. J., *Fed. Proc.*, 1946, **5**, 246.

<sup>11</sup> Spratt, N. T., *J. Exp. Zool.*, 1947, **106**, 345.

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### 17232. Effects of 4-Amino-Pteroylglutamic Acid in Dogs with Special Reference to Megaloblastosis.\*

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#### Experimental production of macrocytic

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anemia and megaloblastosis of bone marrow has been achieved by administration of diets deficient in folic acid (PGA). Miller and Rhoads<sup>1</sup> observed in dogs fed the Goldberger

<sup>1</sup> Miller, D. K., and Rhoads, C. P., *Proc. Soc. Exp. Biol. and Med.*, 1933, **30**, 540.

diet macrocytic anemia with megaloblasts in the purple marrow, leucopenia and diarrhea. The same authors described later a hypercellular, megaloblastic marrow in pigs receiving a similar diet.<sup>2</sup> Wills and Stewart<sup>3</sup> reported an experimental macrocytic anemia with megaloblasts in bone marrow in their classical studies with monkeys fed purified diets. In the light of present knowledge the diets employed in the above studies were probably deficient in PGA.<sup>4-9</sup> Subsequent studies in pigs receiving PGA-deficient diets supplemented with a folic acid antagonist, x-methyl-PGA, and sulfasuxidine provided further evidence of the role of folic acid in the maintenance of hematopoiesis. The addition of x-methyl-PGA enhanced the development of macrocytic anemia and leucopenia which responded rapidly to PGA and its conjugates. The bone marrow of deficient pigs showed hyperactive erythropoiesis with megaloblastosis similar to that seen in pernicious anemia of man in relapse.<sup>10,11</sup> With the synthesis of 4-amino-pteroylglutamic acid (4-amino-PGA)<sup>12</sup> an antagonist was provided which proved more potent and more rapid in inducing signs of folic acid deficiency in mice

and rats<sup>13-16</sup> than x-methyl-PGA. In view of this finding it was of interest to determine whether the administration of 4-amino-PGA to dogs would also elicit signs of PGA-deficiency similar to the syndrome described by Miller and Rhoads.<sup>1</sup>

**Procedure.** Seventeen adult, mongrel dogs were used (Table I). The antagonist<sup>‡</sup> was prepared for daily use by dissolving with 2 molar equivalents of NaHCO<sub>3</sub> or NaOH to achieve a final pH of less than 8.0. Complete hematological examination including volumetric estimations<sup>17</sup> of blood drawn from the jugular vein into heparinized syringes and of sternal bone marrow aspirations were carried out prior to administration of 4-amino-PGA and at intervals thereafter. The animals were anesthetized briefly with 10 to 20 mg/kg pentothal sodium, intravenously, to facilitate sampling of bone marrow. Blood and marrow films were stained in Jenner-Giemsa buffered at pH 6.85. At autopsy marrow was collected from sternum and femur, smeared, and sectioned. Tissues were taken from all internal organs except nervous system, fixed in Vandergrift's, sectioned, and stained with H and E and Giemsa. In 10 dogs plasma chloride<sup>18</sup> and total nitrogen and NPN in trichloroacetic acid filtrates using the micro-Kjeldahl procedure and blood glucose<sup>19</sup> were followed. Plasma protein was calculated to be 6.25 times protein nitrogen.

**Clinical course.** In dogs 1 through 9 (Table I) receiving single, large doses of 4-amino-PGA no adverse signs were noted until after

<sup>2</sup> Miller, D. K., and Rhoads, C. P., *J. Clin. Invest.*, 1935, **14**, 153.

<sup>3</sup> Wills, L., and Stewart, A., *Brit. J. Exp. Path.*, 1935, **16**, 444.

<sup>4</sup> Jukes, T. H., and Stokstad, E. L. R., *Physiol. Rev.*, 1948, **28**, 51.

<sup>5</sup> Langston, W. C., Darby, W. J., Shukers, C. F., and Day, P. L., *J. Exp. Med.*, 1938, **68**, 923.

<sup>6</sup> Day, P. L., Langston, W. C., Darby, W. J., Wahlin, J. G., and Mims, V., *J. Exp. Med.*, 1940, **72**, 463.

<sup>7</sup> Day, P. L., Mims, V., and Totter, J. R., *J. Biol. Chem.*, 1945, **161**, 45.

<sup>8</sup> Wilson, H. E., Saslow, S., and Doan, C. A., *J. Lab. Clin. Med.*, 1946, **31**, 631.

<sup>9</sup> Rinehart, J. F., and Greenberg, L. D., *Am. J. Path.*, 1948, **24**, 710.

<sup>10</sup> Cartwright, G. E., Tattling, B., Ashenbrucker, H., and Wintrobe, M. M., *Blood*, 1949, **4**, 301.

<sup>11</sup> Heinle, R. W., Welch, A. D., and Pritchard, J. A., *J. Lab. Clin. Med.*, 1948, **33**, 1647.

<sup>12</sup> Seeger, D. R., Smith, J. M., Jr., Holtquist, M. E., *J. Am. Chem. Soc.*, 1947, **69**, 2567.

<sup>13</sup> Franklin, A. L., Stokstad, E. L. R., and Jukes, T. H., *Proc. Soc. Exp. Biol. and Med.*, 1948, **67**, 398.

<sup>14</sup> Oleson, J. J., Hutchings, B. L., and Subbarow, Y., *J. Biol. Chem.*, 1948, **175**, 359.

<sup>15</sup> Swendseid, M. E., Wittle, E. L., Moersch, G. W., Bird, O. D., and Brown, R. A., *Fed. Am. Soc. Exp. Biol., Proc.*, 1948, **7**, 299.

<sup>16</sup> Philips, F. S., and Thiersch, J. B., *J. Pharm. Exp. Therap.*, 1949, **95**, 303.

<sup>‡</sup> The authors are indebted to the Lederle Laboratories Division and the Calco Division of the American Cyanamid Company for liberal supplies of 4-amino-PGA.

<sup>17</sup> Wintrobe, M., "Clinical Hematology," 2nd ed., Lea and Febiger, Philadelphia, Pa., 1946.

<sup>18</sup> Van Slyke, D. D., and Hiller, A., *J. Biol. Chem.*, 1947, **167**, 107.

<sup>19</sup> Nelson, N., *J. Biol. Chem.*, 1944, **153**, 375.



group attached to such a substance, whose effects are minimized by dilution. One point in Fischer's data might lend support to the idea of such a toxic factor. Fischer reports that either egg albumin or insulin, when added to dialyzed plasma, will protect cells placed thereon from histolytic disintegration. He attributes this effect to the nutritive properties of the labile S-S and S-H bonds in these materials, but both egg albumin and insulin are known to be highly active adsorbents capable of detoxifying various media. This appears to be the effect of serum albumin when added to media for the cultivation of bacteria<sup>10</sup> and may have been a factor in Spratt's experiments<sup>11</sup> with explanted early chick blastoderms (not tissue cultures). It seems quite possible that the beneficial effect of egg albumin or insulin when added to dialyzed plasma may be of similar origin. On the other hand, the defect might be due to an imbalance of nutritive materials. The plasma and embryo juice were dialyzed against a Ringer's solution containing 0.1% dextrose but lacking phosphate. The diluent used in preparing media (Tyrode's solution) contained no dextrose but did contain phosphate. The diluent was thus *not* in equilibrium with the dialyzed materials, and would both extract dextrose therefrom and

add phosphate thereto. If 0.1% dextrose was too high a concentration for fibroblast growth, the defect would be corrected by dilution. Since phosphate is required for growth, the defect might be a simple deficiency as postulated by Fischer, but not necessarily a deficiency of organic materials. However, Fischer's evidence, that a suitable amino-acid mixture prepared with physiological salt solution rather than Tyrode's solution does, in fact, partially correct the defect of dialyzed plasma-embryo juice, shows that phosphate was not the critical factor. It shows also that the low molecular weight materials removed by dialysis, such as the amino acids, are of great and at this level critical importance.

All of this evidence, however, still leaves unresolved the question of the nature of the postulated positive toxic defect in the dialyzed materials. In any case, our observations, though limited, suggest that it is unsafe to assume that dialyzed plasma or embryo juice is truly inert. Until further experiments have been carried out to clarify the effects of dilution on dialyzed extract, we feel that these materials should not be used in the preparation of substrata on which to test the nutritive value of supernatants. We believe that bare glass or cellophane are still the only really safe bases for such studies.

<sup>10</sup> Davis, B. D., and Dubos, R. J., *Fed. Proc.*, 1946, 5, 246.

<sup>11</sup> Spratt, N. T., *J. Exp. Zool.*, 1947, 103, 345.

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<sup>1</sup> Miller, D. K., and Rhoads, C. P., *Proc. Soc. Exp. Biol. and Med.*, 1933, 30, 540.



TABLE I.  
Effect of 4-Amino-PGA on Cells in Blood and Bone Marrow of Dogs.

D. g	Dosage		Day	Wt. kg	Herit., %	PMN	MNC	Differential count of nucleated cells in bone marrow				Differential count of nucleated erythroid cells in bone marrow			
	mg/kg/day	No. of inj.						Myeloid, %	Lymphoid, %	Erythroid, %	Nuclear remnants, %	Normo- blasts, %	Erythro- blasts, %	Megalo- blasts, %	Primitive stem cells, %
1	56iv	1	0 38	18.0 16.3	45.0 52.0	6,950 700	1,650 250	38 32 19F	5 5 2F	57 63 79F	10 29 50F	90 50 23F	0 11 10F	0 10 17F	0 0 0F
2	37iv	1	0 38	16.2 14.8	28.2 33.0	14,200 2,150	2,500 300	47 29F	10 21F	43 50F	10 51F	90 24F	0 0F	0 25F	0 0F
4	20iv	1	0 38	14.1 11.7	44.3 60.0	6,700 650*	550	39 69	3 3	8 28	6 15	90 34	4 10	0 41	0 0
5	20iv	1	0 38	12.2 11.1	32.9 47.0	12,900 4,150	1,900 200	69 78 18F	8 11 8F	23 11 74F	18 26 51F	80 62 28F	2 4 2F	0 8 19F	0 0 0F
6	20iv	1	0 38	12.1 10.9	32.5 37.0	14,600 4,000	950 450	58 78 54F	3 11 8F	44 11 38F	15 26 32F	80 54 11F	5 4 7F	0 8 44F	0 0 6F
7	10iv	1	0 48	13.0 10.2	46.0 58.0	13,100 900	1,600 300	65 23	6 pr	29 77	3 29	96 44	1 2	0 25	0 0
8	10iv	1	0 3	20.9 18.3	41.0 46.7	14,600 5,750	1,100 650	66 68	8 0	26 23	2 28	98 43	0 8	0 21	0 0
9	5iv	1	0 38	16.6 16.7	60.5 29.2	1,850 12,400	100 2,350	45 72 83	2 4 7	53 24 10	24 10 31	40 90 43	4 0 16	0 0 10	32 0 0
10	0.1im	5	0 58	10.5 8.5	47.0 60.5	19,800 9,600	1,250 2,100	80 28	7 15	13 57	6 17	92 53	2 0	0 0	0 30
12	0.1im	5	0 58	10.1 7.9	36.5 58.0	16,600 1,600	4,200 550	40 38	3 7	57 55	5 39	94 36	1 0	0 25	0 0
13	0.05im 0.1im	14 8	0 14 21 24S	11.3 11.8 10.6 9.1	30.1 39.0 43.0 50.0	10,400 14,200 7,350 3,700	650 750 400 100	46 39 82 87F	7 6 2 3F	47 65 16 10F	13 3 28 55F	86 97 72 45F	1 0 0 0F	0 0 0 0F	0 0 0 0F

in intoxication and fully explains the bloody diarrhea which was evident 1 to 2 days prior to sacrifice and before hematocrit values were significantly elevated in dogs receiving acutely lethal doses. Ileitis and colitis in the present animals resembled findings in rats in which microscopic evidence of intestinal damage was detected as early as 6 hours after poisoning with fatal doses although death did not ensue for 3 to 4 days.<sup>16</sup> The pathology of the gut was judged to be more extensive and severe than the focal ulceration known to occur in dogs dying of shock.<sup>20-22</sup> Furthermore, unlike the secondary sequellae of local vasoconstriction in shocked animals, focal ulceration did not occur in duodenum, stomach, or esophagus.

With the exception of dogs 14 and 16 no other lesions of significance were detected in other internal organs. The exceptional animals manifested the effects of ascending infections of probable origin in their damaged intestines.

**Discussion.** The observations described above provide further evidence that 4-amino-PGA acts as an antagonist of PGA and, thereby, confirm previous deductions from studies in rats and mice.<sup>16</sup> The syndrome produced in dogs of ileitis and colitis with diarrhea, peripheral leucopenia, and depletion of bone marrow with appearance of megaloblasts corresponds to observations of the effects of folic acid deficiency in dogs, pigs, and monkeys. The diarrhea and changes in hematopoiesis resemble sprue in man, a disease responding readily to PGA therapy (see recent review<sup>4</sup>).

A comparison of bone marrow from dogs receiving 4-amino-PGA with marrow from cases of pernicious anemia in man reveals certain features in common. These include increased numbers of hypersegmented polymorphonuclears and giant metamyelocytes, megaloblasts and irregularly shaped nuclear remnants in erythrocytes. However, differences between the two marrows are apparent. In

pernicious anemia bone marrow exhibits uniform hyperplasia of both erythroid and myeloid elements. In the present dogs general cellularity was reduced and confined to islands of active marrow. In untreated pernicious anemia erythropoiesis is predominantly megaloblastic whereas in the present animals erythropoiesis was a mixture of normoblastic and megaloblastic elements thus corresponding to marrow's reported in sprue.<sup>23-28</sup>

The megaloblasts produced in dogs resembled those found in pernicious anemia and sprue as well as those found in humans treated with 4-amino-PGA<sup>29</sup> possibly more closely than the megaloblastic-like cells observed in swine receiving either PGA-deficient diets supplemented with x-methyl folic acid<sup>10</sup> or injections of 4-amino-PGA.<sup>30,31</sup> From studies now available it would appear that megaloblastic response of erythropoiesis to PGA-deficiency varies in different species. Megaloblasts have not been observed in mice and rats,<sup>16,32,33</sup> with the exception of a single report.<sup>34</sup>

**Summary.** The administration of 4-amino-PGA to dogs produced a sprue-like syndrome with diarrhea, peripheral leucopenia, depletion of bone marrow with increased numbers of hypersegmented polymorphonuclears and giant metamyelocytes, abnormal nuclear dis-

<sup>23</sup> Krjukoff, A., *Folia Haematol.*, 1928, **35**, 329.

<sup>24</sup> Ashford, B. K., *Am. J. Trop. Med.*, 1932, **12**, 199.

<sup>25</sup> Makic, F. P., and Fairley, N. H., *Indian J. Med. Res.*, 1929, **16**, 799.

<sup>26</sup> Castle, W. B., and Rhoads, C. P., *Arch. Int. Med.*, 1935, **56**, 627.

<sup>27</sup> Hotz, H. W., and Rohr, K., *Erg. Inn. Med. u. Kindheilk.*, 1938, **54**, 174.

<sup>28</sup> Rodriguez, M., *Puerto Rico J. Pub. Health Trop. Med.*, 1939, **15**, 89.

<sup>29</sup> Thiersch, J. B., *Cancer*, 1949, **2**.

<sup>30</sup> Cartwright, G. E., and Wintrobe, M. M., personal communication.

<sup>31</sup> Thiersch, J. B., and Philips, F. S., unpublished observations.

<sup>32</sup> Endicott, K. M., Daft, F. S., and Ott, M., *Arch. Pathol.*, 1945, **40**, 364.

<sup>33</sup> Weir, D. R., Heinle, R. W., and Welch, A. D., *Proc. Soc. Exp. Biol. and Med.*, 1948, **69**, 211.

<sup>34</sup> Franklin, A. L., Stokstad, E. L. R., Belt, M., and Jukes, T. H., *J. Biol. Chem.*, 1947, **169**, 427.

<sup>20</sup> Blalock, A., *Arch. Surg.*, 1934, **29**, 837.

<sup>21</sup> Klemperer, P., Penner, A., and Bernheim, A. L., *Am. J. Digest. Dis.*, 1940, **7**, 410.

<sup>22</sup> Moon, V. H., Shock. Its dynamics, occurrence and management, Lea and Febiger, Philadelphia, 1942.



FIG. 1.

Bone marrow aspiration of dog 7 four days after 10 mg/kg of 4-amino-PGA, intravenously. Two megaloblasts, a nuclear "explosion," and a normoblast with nuclear remnant are included in field.

arate differential counts of nucleated erythroid cells are listed in Table I.

*Lesions in myeloid tissues.* Peripheral granulocytopenia was associated with depletion of metamyelocytes and neutrophils in bone marrow. However, myelocytes and myeloblasts were uniformly present often maintaining their initial relative proportions in spite of general depletion of myelopoiesis. Only dogs 14 and 16 evidenced active myeloid regeneration. In some cases nests of eosinophils were found in depleted marrows similar to previous findings in rats<sup>16</sup>—an observation still unexplained. Of additional interest was the appearance of giant metamyelocytes, not present prior to administration of 4-amino-PGA, and an increased proportion of hypersegmented neutrophils.

Thrombocytoblasts decreased in numbers with general marrow depletion but were not specifically affected.

*Lesions in erythroid tissues.* Within 24 to 48 hours after large single doses numerous mature normoblasts were seen to be in pathological mitosis with disintegration of nuclei, chromosomal segregation, and chromatin expulsion into the cytoplasm often simulating nuclear explosions. This process resulted in increased numbers of erythrocytes with nuclear remnants (Fig. 1). During the same period many of the remaining normoblasts and erythroblasts underwent a change in nuclear pattern. The naturally lumpy chromatin was transformed into a distinct network. In the more primitive erythroid elements with basophilic cytoplasm a purple parachromatin appeared consistent with the structure of

megaloblasts. Later even hemoglobinized cells with parachromatin were observed. After 48 to 72 hours the proportion of normoblasts was much reduced. In dog 6, for example, at this stage, 44% of nucleated erythroid elements in femoral marrow showed nuclear parachromatin and 32%, nuclear remnants. Later with continued depletion primitive stem and sinus endothelial cells become prominent in smears and sections (dogs 6 and 8).

In dogs 10 through 17 megaloblasts were observed in only 3 cases. However, depletion of hematopoiesis was extreme in all dogs receiving repeated injections and megaloblasts might have been present at some stage of intoxication when marrow samples were not taken. The lesions found in dogs 10 through 17 were otherwise identical to those described above.

Lymphoid tissues of spleen, mesenteric nodes, and intestinal tract appeared to be reduced in quantity but no pyknosis or necrosis was seen. By comparison to depleted bone marrows lymphoid tissues were relatively unaffected.

*Lesions of the digestive tract* were confined to jejunum, ileum, and colon. In all animals at autopsy marked edema and swelling of the mucosa was found throughout small and large intestines. Villi were thickened and blunted by capillary dilatation near the tips. The cytoplasm of superficial epithelial cells was enlarged. In almost the entire colon and ileum epithelium had desquamated leading to denudation of villi and superficial ulceration. In glands and crypts there was evidence of cytoplasmic enlargement and desquamation of epithelium with abnormal regeneration of cells containing irregular nuclei and cytoplasm. Lumina of the deeper glands were frequently filled with plugs consisting of desquamated epithelium and leucocytes. The villi appeared to be matted together resulting from loss of superficial portions and infiltration with leucocytes and fibroblasts below ulcerated areas.

The widespread involvement and necrosis can in large part be attributed to a primary action of 4-amino-PGA on intestinal mucosa. Intestinal damage probably developed early

TABLE I.  
Mortality and Growth Rates of Rats Receiving  $\gamma$ -BHC.

Supplement		No. rats	% mortality	Growth, g/wk (avg)
Inositol, mg/g	$\gamma$ -BHC, mg/g			
—	—	24	0	28.0
0.5	—	12	0	26.6
2.0	—	8	0	28.9
0	0.8	12	75	11.9
0.5	0.8	24	63	17.1
2.0	0.8	18	72	18.2
0	0.4	16	6	26.0
0.5	0.4	22	36	27.4
2.0	0.4	24	8	28.2

TABLE II.  
Mortality and Growth Rates of Littermate Rats Receiving  $\gamma$ -BHC.

Group	Supplement		No. rats	% mortality	Growth rate, g gain/wk		
	Inositol, mg/g	$\gamma$ -BHC, mg/g			1	2	Avg 4 wks
1	—	—	22	0	24.3	29.6	30.1
2	0.5	—	22	0	25.9	27.6	30.1
3	2.0	—	22	0	24.8	30.0	29.8
4	4.0	—	22	0	26.6	29.8	31.3
5	—	0.4	22	14	10.6	27.6	25.4
6	0.5	0.4	22	14	14.1	29.8	27.9
7	2.0	0.4	22	18	11.6	29.4	27.1
8	4.0	0.4	22	36	15.2	29.7	28.7

ide, 0.4; fat-soluble vitamin mixture,<sup>11</sup> 2.0; lard, 8.0; sucrose, 67.3%.

Supplements of  $\gamma$ -BHC were dissolved in melted lard, while inositol was added at the expense of sucrose. Four week feeding periods were studied throughout. Results of preliminary experiments are presented in Table I.

Control animals on the suboptimal (15%) protein levels gained weight at a rate of 26.6-28.9 g per week. Since the animals receiving no dietary inositol grew as well as those receiving adequate quantities of inositol, it may be stated that with these rations this strain

of rat requires no dietary inositol. The presence of  $\gamma$ -BHC in these diets led to markedly diminished growth rates and significant mortality rates, particularly during the first two weeks. Addition of inositol seemed to be without effect. No alopecia, spectacled eye, or other possible signs of inositol deficiency were observed in animals maintained on inositol-free diets for experimental periods up to 8 weeks.

The sequence of events following the administration of sufficient  $\gamma$ -BHC is striking; within 24-48 hours the temperament of the animals alters from docility to ferocity; they react explosively to tactile stimuli, leaping, squealing, and biting as if in pain when touched lightly on the back. They seem to be less sensitive to auditory stimuli, but, superficially, the picture resembles somewhat that seen in magnesium deficiency.<sup>8</sup> Generalized tonic-clonic convulsions are frequent,

<sup>11</sup> B-Complex mixture: Ca pantothenate, 5.0; niacinamide, 2.5; para-aminobenzoic acid, 1.0; riboflavin, 1.0; pyridoxine · HCl, 0.5; thiamine · HCl, 0.5; biotin, 0.1; "Polyvit," 0.05; and sucrose, 989.35 g.

<sup>1</sup> Fat-soluble vitamin mixture: Peanut oil, 195.0;  $\alpha$ -tocopherol acetate, 5.0 g. Forty g of this solution is added to cod liver oil, 2.0; corn oil (Mazola), 157.5; 2-methyl-1,4-naphthoquinone, 0.5 g.

<sup>8</sup> Kruse, H. D., Orent, E. R., and McCollum, E. V., *J. Biol. Chem.*, 1932, **90**, 519.

integration of normoblasts, change of nuclear pattern in erythroid elements and megablasts. Doses given were lethal and animals succumbed with anorexia, weight loss, dehydration, and hemorrhagic diarrhea following

severe ileitis and ulcerative colitis. It is suggested that the syndrome results from antagonism of folic acid.

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### 17233. Chronic Toxicity of Gamma Isomer of Hexachlorocyclohexane in the Albino Rat.

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Included in the report by Slade<sup>1</sup> on the discovery of the potent insecticide, "Gam-mexane," the gamma isomer of benzene hexachloride ( $\gamma$ -BHC)\* were investigations of the acute and chronic toxicity of the drug in laboratory rats. His report indicates that the gamma isomer is much more toxic than the  $\alpha$ ,  $\beta$ , or  $\delta$  isomers on oral administration, but, when mixed with the diet, levels of  $\gamma$ -BHC up to 30 mg per rat per day had no effect over a 5 week period. Other acute experiments have demonstrated by oral, subcutaneous and intravenous administration, the toxicity of  $\gamma$ -BHC,<sup>2,3</sup> while Laug<sup>4</sup> reported that the gamma isomer was non-toxic when fed in the diet at 20, 500, and 1000 p.p.m. for periods up to 114 days.

Other workers have been interested in the anti-inositol properties of the gamma isomer, since it was reported to resemble the structure of inositol.<sup>1</sup> Their results indicate that the inhibitory effect of  $\gamma$ -BHC is reversed by

inositol in the growth of a microorganism,<sup>5</sup> in the  $\alpha$ -amylase enzyme system of the pancreas,<sup>6</sup> and in the growing onion root.<sup>7</sup>

A study of transmethylation and lipotropic agents stimulated in this laboratory by the work of Griffith indicated that it might be of interest to examine the anti-inositol and toxic properties of the gamma isomer in the rat, possibly to make available another method of studying inositol metabolism in the intact animal.

Weanling male rats of the St. Louis University strain, 21-24 days old, weighing 44-49 g, were housed in screen cages and allowed food and water *ad libitum*. The animals were weighed twice weekly. The diet to which supplements of  $\gamma$ -BHC and inositol were added was as follows: Casein, 15.0; l-cystine, 0.3; salts 3A,<sup>†</sup> 2.0; salts 3B,<sup>‡</sup> 2.0; cellulose, 2.0; B-complex mixture,<sup>§</sup> 1.0; choline chlor-

<sup>6</sup> Lane, R. L., and Williams, R. J., *Arch. Biochem.*, 1948, 19, 329.

<sup>7</sup> Chargaff, E., Stewart, R. N., and Magnusanik, B., *Science*, 1948, 108, 556.

<sup>†</sup> The salt and vitamin mixtures were devised and thoroughly tested by Dr. Wendell H. Griffith. Since the compositions of these supplements have not been published, the authors wish to express their thanks to Dr. Griffith for his permission to present them here. Salts 3A:  $\text{CuSO}_4$ , 0.5;  $\text{CoSO}_4 \cdot 7 \text{H}_2\text{O}$ , 0.1;  $\text{ZnSO}_4 \cdot 7 \text{H}_2\text{O}$ , 0.9;  $\text{MnSO}_4 \cdot 4 \text{H}_2\text{O}$ , 4.0;  $\text{K}_2\text{Al}_2(\text{SO}_4)_3 \cdot 24 \text{H}_2\text{O}$ , 0.07;  $\text{NaF}$ , 0.05; Ferrie citrate, 24.38;  $\text{CaCO}_3$ , 100;  $\text{Ca citrate}$ , 185;  $\text{MgCO}_3$ , 45.0;  $\text{K}_2\text{HPO}_4$ , 140 g.

<sup>‡</sup> Salts 3B:  $\text{KI}$ , 0.8;  $\text{NaCl}$ , 169.2;  $\text{K}_2\text{HPO}_4$ , 100;  $\text{CaH}_2\text{PO}_4$ , 230 g.

<sup>1</sup> Slade, R. E., *Chem. Ind.*, 1945, 64, 314.

\* The term, gamma benzene hexachloride, has been approved by the Committee on Insecticide Nomenclature as the accepted name for the gamma isomer of hexachlorocyclohexane (*Science*, 1949, 109, 330).

<sup>2</sup> Cameron, G. R., and Burgess, F., *Insecticide Development Panel Report (44)*, 1944, 131.

<sup>3</sup> McNamara, B. P., and Krop, S., *J. Pharm. Exp. Therap.*, 1948, 92, 140.

<sup>4</sup> Laug, E. P., *J. Pharm. Exp. Therap.*, 1948, 93, 277.

<sup>5</sup> Kirkwood, S., and Phillips, P. H., *J. Biol. Chem.*, 1946, 163, 251.

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Gemmill<sup>1</sup> first demonstrated that insulin stimulated glycogen deposition from glucose in isolated rat diaphragm. Subsequent study with this tissue has disclosed the following important points. Low potassium<sup>2</sup> and high glucose<sup>1</sup> concentrations favor glycogen synthesis. The insulin effect can be demonstrated in diaphragm from either adrenalectomized or hypophysectomized<sup>3</sup> rats and so apparently is independent of the action of hormones from

these sources. Desoxycorticosterone, corticosterone and some related substances give reduced glycogen levels when incubated with diaphragm muscle, the effect appearing to be of a glycogenolytic nature and separate from the glycogenic response to insulin.<sup>4</sup>

We have recently examined glucose metabolism in the rat diaphragm with the help of radioactive C<sup>14</sup> glucose.<sup>5</sup> An increase in the radioactivity of the glycogen in the presence of insulin closely proportionate to the increased glycogen content by analysis provided additional evidence that glucose was the direct precursor of the glycogen rather than a stimulant of glycogenesis from other sources. Insulin pro-

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accompanied by hyperpnea and later by hypopnea and exhaustion (some aspects of this syndrome were observed in acute toxicity experiments with  $\gamma$ -BHC<sup>2,3</sup>). Some animals having 2 or more convulsions during the first week have subsequently recovered completely. Of weanling animals failing to survive, death occurred from the second to tenth experimental days.

Additional experiments examined the toxic effects of levels of dietary  $\gamma$ -BHC at 0.6 and 0.2 mg per gram of food. The higher level was lethal to a large proportion of animals, while the lower level was non-toxic and without effect on growth rates. Thus the optimal level of  $\gamma$ -BHC for study was selected at 0.4 mg/g of food; in a similar experiment litter mate controls were used throughout the groups. Growth rates and mortality data are presented in Table II.

The data again demonstrate that this strain of rat does not require supplemental inositol under these experimental conditions. The addition of 0.4 mg of  $\gamma$ -BHC to the diets reduced growth rates markedly during the first week, but during the succeeding 3 weeks, growth rates of surviving animals receiving  $\gamma$ -BHC compared favorably with those of the controls, indicating the development of tolerance. The addition of increasing amounts of inositol seemed to combat slightly the growth depressant effect of  $\gamma$ -BHC (group 5 compared with groups 6, 7 and 8), but had also the effect of increasing mortality. The latter may have been due to a slight increase in food consumption during the first week with a slightly greater intake of  $\gamma$ -BHC in those animals receiving higher levels of inositol. (The first week the controls averaged 7.2 g of food per rat per day, while the food intakes of the animals receiving  $\gamma$ -BHC were: group 5, 4.9; group 6, 5.1; group 7, 5.1; and group 8, 5.3 g of food per rat per day.)

In other experiments the effect of 0.8 mg  $\gamma$ -BHC per gram of food was tested in weanling rats fed a stock laboratory ration, i.e., ground Purina lab chow, to ascertain if the type of diet influenced the reaction to the drug. The young animals exhibited hyperirritability, hyperactivity, convulsions, retarded growth, and a mortality of 25%. Adult

rats raised on chow, when placed on the basal ration=plus- $\gamma$ -BHC-at-the 0.8 mg level, lost 10% of their body weight over 10-14 days; in the succeeding two weeks they returned to their starting weights and continued a slow gain. Irritability and infrequent convulsions without mortality resulted.

Animals raised on the highly purified ration (inositol-free) to body weights of 200-300 grams, showed typical symptomatology with a mortality of 50% when the gamma isomer was added to the ration at the level of 0.8 mg per g of food. These animals obtained 3.4-4.1 mg of  $\gamma$ -BHC per rat per day while the weanling rats dying (Table II) received between 1.8-3.1 mg. (Adult rats dying ate 188 mg/kg over a 10-day average survival time, while weanling rats consumed 200 mg/kg body weight over a 4-day average survival period.)

These results are not in accord with those of Slade<sup>1</sup> and Laug<sup>4</sup> whose experimental diets were not reported; Slade reported  $\gamma$ -BHC to be non-toxic when mixed with the diet in amounts up to 30 mg per rat per day, while calculation of the data presented by Laug reveals no toxic effects with dosages up to 20 mg per day. Recently Fitzhugh *et al.*<sup>9</sup> reported that the gamma isomer, when mixed with the diet, is toxic at the level of 0.8 mg per gram of food.

The authors wish to point out that stress was placed upon the rats used in these experiments by suboptimal dietary protein levels in an effort to enhance the action of  $\gamma$ -BHC. Further, animals raised on stock rations tolerated diets containing  $\gamma$ -BHC much better than those raised on experimental diets. These discrepancies may in part be due to the higher nutritive value of normal stock rations, as compared with highly purified rations.

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method.<sup>11</sup> 100 - 120 g female albino rats were used and that portion of each internal oblique muscle which could be separated as a thin layer without appreciable trauma was placed intact in the incubating flask. When the muscle was cut into a series of thin strips, little or no glycogen synthesis took place.

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**Summary.** If used in thin sheets with a minimum of tissue injury isolated skeletal muscle in the form of the internal oblique muscle of the rat forms more glycogen under the influence of insulin just as does the specialized muscle comprising the diaphragm. Desoxycorticosterone reduces the amount of glycogen formed by incubation with glucose alone as in the case of diaphragm muscle. Skeletal muscle offers new material for further investigation of the action of insulin.

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### 17235. Normal and Seizure Levels of Lactate, Pyruvate and Acid-Soluble Phosphates in the Cerebellum and Cerebrum.\*

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induced in a series of cats using representative nitrogen mustards and various other convulsants, the brains were frozen *in situ* and the cerebrum and cerebellum then analyzed separately for lactic acid, pyruvic acid, inorganic phosphorus, and high energy phosphates.

**Experimental.** Preparation of the animals, methods of EEG and EKG recording, procedure of freezing with liquid air and methods of analysis have been reported in detail in previous papers from this laboratory.<sup>1,5</sup> Seizures were induced by intravenous injections of the compounds indicated in Table I. Cerebral and cerebellar cortex were easily dissected from the frozen brains with a small chisel and mallet. During the dissection frequent applications of liquid air were used to maintain the tissue in a frozen state.

**Results.** The data are summarized in Table I. In normal brains frozen without seizures the levels of the constituents determined were very similar in both cerebellum and cerebrum and compared well with those previously reported<sup>1,5</sup> except that the phosphocreatine levels were somewhat low. In brains frozen during seizures, however, marked dif-

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TABLE I.  
Effect of Insulin and Desoxycorticosterone on Glycogen in Isolated Rat Abdominal Muscle.

Exp.	Days fasted	Mg of glycogen per 100 mg (wet wt) muscle				
		Initial value	Incubated 2 hr at 38° under O <sub>2</sub>			
			Without glucose	Glucose	Glucose + insulin	Glucose + DOC
1	0	.12	.10	.29	.50	.16
2	0	.16	.19	.35	.54	.30
3	0	.22	.13	.33	.42	.18
4	0	.39	.32	.52	.62	.35
5	0	.22	.21	.37	.59	.36
6	0	.28	.16	.40	.50	.22
7	1	.21	.14	.29	.52	.21
8	1	.15	.16	.32	.38	.17
9	1	.24	.15	.44	.65	.24
10	2	.12	.16	.30	.48	.16
11	2	.21	.14	.32	.45	.20
12	3	.25	.10	.26	.44	.23
Avg		.21	.16	.35	.51	.23

duced a small but significant increase in the radioactive CO<sub>2</sub>, hence an acceleration of the combustion of the sugar. Desoxycorticosterone and corticosterone antagonized glycogen formation without appreciably influencing glucose utilization or respiration values and without increasing radioactive carbon dioxide.

The use of insulin in general with isolated tissue slice, mince or extracts, has proved discouraging. Few verifiable effects of any magnitude have been published other than the rat diaphragm work. There has been considerable interest in the reports from Cori's laboratory suggesting that insulin can release some sort of combined pituitary-adrenal cortical inhibition of hexokinase,<sup>6,7</sup> the enzyme responsible for the first step in the utilization of glucose; and unified hypotheses covering the hexokinase action have been offered to explain insulin action *in vivo*.<sup>8</sup> Unfortunately the hexokinase experiments have been ambiguous and difficult to repeat and there appears to be some doubt as to whether even one of the points of action of insulin is a hexokinase inhibitory release.<sup>9,10</sup>

Diaphragm muscle is an important tissue but a specialized muscle with a limited resting period and undergoing continuous rhythmic contractions throughout life. On exploring the possibility of using tissue other than the diaphragm for insulin effects, we have generally found that chopped, shredded or teased muscle loses its ability to synthesize glycogen from glucose. However, we have been able to dissect the internal oblique abdominal muscle of the rat with a minimum of tearing and find that this muscle *in vitro* will give considerable glycogen synthesis from added glucose and also a further large increase of glycogen in the presence of very small amounts of insulin. As in the case of the diaphragm less glycogen is found in the presence of desoxycorticosterone. Results of experiments with this tissue are recorded in Table I. 400 - 500 mg wet weight of abdominal muscle was incubated in 3.0 ml of pH 7.4 Ringer phosphate with or without 0.2 molar glucose, 0.1 unit per ml of insulin and 10 µg per ml of desoxycorticosterone.<sup>†</sup> Flasks were shaken for 2 hours in an oxygen atmosphere. Glycogen was assayed by the usual alkali digestion-alcohol precipitation

<sup>6</sup> Colowick, S. P., Cori, G. T., and Slein, M. W., *J. Biol. Chem.*, 1947, **168**, 583.

<sup>7</sup> Price, W. H., Cori, C. F., and Colowick, S. P., *J. Biol. Chem.*, 1945, **160**, 633.

<sup>8</sup> Stetten, D., Jr., *J.A.M.A.*, 1946, **132**, 373.

<sup>9</sup> Broh-Kahn, R., and Mirsky, I. A., *Science*, 1947, **106**, 148.

<sup>10</sup> Stadie, W. C., and Haugaard, N., *J. Biol. Chem.*, 1949, **177**, 311.

<sup>†</sup> Pure Zn insulin was obtained through the courtesy of Dr. Edwin E. Hays of Armour and Co., and desoxycorticosterone from Dr. Edward Henderson of the Schering Corp.

method.<sup>11</sup> 100 - 120 g female albino rats were used and that portion of each internal oblique muscle which could be separated as a thin layer without appreciable trauma was placed intact in the incubating flask. When the muscle was cut into a series of thin strips, little or no glycogen synthesis took place.

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Sedrum.												
Lactate*		Pyruvate		Inorg. P		Phospho- creatine		Adenosine Tri-PO <sub>4</sub>		Adenosine Di-PO <sub>4</sub>		Remarks
Cb†	Cx	Cb	Cx	Cb	Cx	Cb	Cx	Cb	Cx	Cb	Cx	
Controls.												
1.37	1.36	.069	.077	6.72	5.11	1.58	1.49	0.81	0.48	2.56	2.70	
1.50	1.40	.120	.136	4.70	4.93	2.10	1.95	1.30	0.90	1.85	2.06	
1.43	1.38	.095	.106	5.71	5.02	1.84	1.72	1.06	0.69	2.20	2.38	Avg
Nitrogen Mustards.‡												
1.80	4.39	.177	.213	4.17	4.24	2.10	1.20	0.39	1.69	3.15	1.32	C <sub>7</sub> H <sub>7</sub> N(C <sub>2</sub> H <sub>4</sub> Cl) <sub>2</sub>
3.48	5.86	.193	.268	3.84	3.60	2.28	1.91	1.43	0.85	1.82	2.51	H <sub>2</sub> C=CHN(C <sub>2</sub> H <sub>4</sub> Cl) <sub>2</sub>
1.66	5.64	.134	.208	4.56	4.20	2.29	1.92	1.13	2.25	2.19	1.00	C <sub>6</sub> H <sub>5</sub> CH <sub>2</sub> N(C <sub>2</sub> H <sub>4</sub> Cl) <sub>2</sub>
1.18	2.76	—	—	4.08	4.56	2.77	1.69	1.71	1.49	1.73	1.57	OC <sub>4</sub> H <sub>8</sub> NC <sub>2</sub> H <sub>4</sub> Cl
3.45	4.08	.277	—	5.19	3.58	1.38	2.30	1.58	1.46	1.97	1.60	C <sub>4</sub> H <sub>9</sub> N(C <sub>2</sub> H <sub>4</sub> Cl) <sub>2</sub>
1.86	2.10	.132	.146	5.94	4.56	3.08	2.16	1.59	0.79	2.00	2.10	C <sub>3</sub> H <sub>7</sub> N(C <sub>2</sub> H <sub>4</sub> Cl) <sub>2</sub>
3.82	7.60	.250	.280	3.72	3.48	3.13	2.03	—	1.36	3.15	1.77	(C <sub>2</sub> H <sub>4</sub> Cl) <sub>2</sub> NCH <sub>2</sub> CH <sub>2</sub> N(C <sub>2</sub> H <sub>4</sub> Cl) <sub>2</sub>
2.46	4.62	.194	.227	4.50	4.03	2.43	1.89	1.30	1.41	2.30	1.70	Avg
Metrazol.												
1.37	2.42	.087	.100	5.18	4.83	2.52	1.50	0.38	0.52	3.48	2.73	
1.50	2.66	.104	.110	4.97	4.68	1.87	1.20	0.53	0.71	2.84	2.35	
1.43	2.54	.095	.105	5.07	4.53	2.19	1.35	0.45	0.62	3.16	2.54	Avg
Aminophylline.												
2.76	9.72	.116	.145	5.88	6.54	1.52	0.42	0.42	0.58	3.00	2.96	16' convulsion
1.56	6.06	.154	.293	(8.21	5.88)			0.75	1.10	3.11	2.18	13' "
4.34	4.08	.126	.156	5.90	5.64	0.82	0.42	0.11	0.40	3.02	2.70	8' "
3.96	9.72	.112	.106	5.94	6.74	0.60	0.08	0.33	0.56	2.90	2.74	4' "
3.16	7.40	.127	.175	6.48	6.20	0.99	0.31	0.40	0.66	3.01	2.64	Avg
Various.												
2.64	3.51	.240	.130	4.92	3.96	2.05	1.81	1.42	2.28	2.19	0.97	Castorix
1.62	3.48	.126	.164	(6.48	5.62)§			1.62	1.52	1.85	1.78	Dibenamine
2.68	4.50	.188	.254	(6.72	5.36)			1.16	1.64	2.24	1.42	Br. Camphor
5.74	6.04	.259	.339	(6.12	5.02)			—	—	—	—	Coramine
2.74	3.60	.164	.206	(7.10	5.18)			1.44	1.16	2.18	1.74	Strychnine
2.20	4.08	.115	.156	3.90	4.14	2.46	2.22	0.92	0.80	1.86	2.30	Electric shock

\* All values mM/1000 g (wet wt).

† "Cb" and "Cx" designate cerebellar and cerebral cortex, respectively.

‡ Obtained through the courtesy of the University of Chicago Toxicity Laboratory.

§ Pairs of figures enclosed in parentheses are the sum of inorganic phosphate and phosphocreatine for cerebellum and cerebrum, respectively.

|| 2-Chloro 4-dimethyl 1,6-methyl pyrimidine.

ferences were found. The lactic acid level rose only about half as much and the phosphocreatine level was appreciably higher in the cerebellum as compared to the cerebrum. There were no systematic differences discernible in the inorganic phosphorus and ATP-ADP levels; however, this may be due to the fact that many of the seizures were potentiated with carbon dioxide<sup>4</sup> a procedure which tends to minimize changes in the assayed constituents during seizures.<sup>5</sup> These results ob-

tained despite the fact that seizure activity was as violent in the cerebellum as in the cerebrum as judged from the EEG recordings.

**Discussion.** The results reported above point to a quantitative if not qualitative difference in the metabolism of the cerebellum and the cerebrum under the stress of seizures. It is not surprising that this should be so since there are marked histological differences between the two parts. Furthermore, the various parts of the central nervous system

have been shown to differ in their sensitivity to cyanide,<sup>6</sup> their carbonic anhydrase activity,<sup>7</sup> their cholinesterase activity,<sup>8</sup> and many other particulars.

**Summary.** Lactic acid, pyruvic acid, inorganic phosphorus, phosphocreatine, and ATP-ADP were determined in liquid air fixed

brains of normal and convulsed cats. In normal brains the levels in cerebellum and cerebrum were approximately the same. In convulsed brains, however, the lactic acid increases were much smaller and phosphocreatine levels were higher in cerebellum than in cerebrum despite the fact that seizure activity was approximately of equal intensity in both parts.

The authors wish to thank Miss Ruth Hurwitz for valuable technical assistance.

<sup>6</sup> Ward, A. A., and Wheatley, M. D., *J. Neuro-path. and Exp. Neurol.*, 1947, **6**, 292.

<sup>7</sup> Ashby, W., *J. Biol. Chem.*, 1944, **152**, 235.

<sup>8</sup> Augustinsson, K. B., *Acta Physiologica Scandinavica*, 1948, **15**, suppl. 52, 8.

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### 17236. Lactate, Pyruvate, and Acid-Soluble Phosphates in Monkey Brains Treated with Carbon Dioxide and Electric Shock.\*

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(Introduced by Warren S. McCulloch.)

*From the Departments of Pharmacology and Psychiatry, University of Illinois, College of Medicine, Chicago, Ill.*

There now exists in the literature a considerable body of data regarding the effect of seizures upon the lactate, pyruvate, and acid-soluble phosphate levels in the brains of dogs,<sup>1</sup> rats,<sup>2</sup> and cats<sup>3,4,5</sup> under various conditions. It seemed, therefore, desirable to extend these analyses to animals higher in the phylogenetic scale, including man. As a step toward this end, a limited series of monkeys has been studied in the normal state, treated with carbon dioxide,<sup>4</sup> electric shock, and electric shock plus carbon dioxide.<sup>6</sup>

**Methods and Results.** Treatment of the animals, brain wave recordings, and analysis of the liquid air fixed brains were carried out as described in previous reports from this

laboratory.<sup>3-6</sup> The results are summarized in Table I. Comparison with the data on other animals shows that the levels of all constituents assayed are of the same magnitude in monkeys as in dogs, cats, and rats and the changes brought about by seizures and carbon dioxide inhalation are in the expected direction.

**Discussion.** The results reported show that it is probably justifiable to extend conclusions drawn from data on experimental animals to man, as far as the brain constituents here assayed are concerned, since it seems unlikely that there would be a sharp change between man and monkey when monkey does not differ from other mammals.

Another point which is clearly demonstrated by a comparison of animals 3 and 4 is that the rise in lactate, pyruvate, and inorganic phosphate elicited by seizures is the result of the hyperactivity of the brain and not of the stimulus which initiates said hyperactivity. There is an indication in the data that the directly driven response to the stimulus alone, without seizures, may cause a fall in phosphocreatine (animal 4) and possible adenosine triphosphate; but in the case of the latter

\* Aided in part by grants from the Miller Epilepsy Fund and the Rockefeller Foundation.

<sup>1</sup> Stone, W. E., Webster, J. E., and Gurdjian, E. S., *J. Neurophysiol.*, 1945, **8**, 233.

<sup>2</sup> LePage, G. A., *Am. J. Physiol.*, 1946, **146**, 267.

<sup>3</sup> Klein, J. R., and Olsen, N. A., *J. Biol. Chem.*, 1947, **167**, 747.

<sup>4</sup> Bain, J. A., and Klein, J. R., *Am. J. Physiol.*, Sept., 1949, **158**.

<sup>5</sup> Bain, J. A., and Pollock, G. H., in preparation.

<sup>6</sup> Stein, S. N., and Pollock, G. H., *Proc. Soc. Exp. Biol. and Med.*, 1949, **70**, 290.



TABLE I.  
Normal and Seizure Levels of Lactate, Pyruvate and Acid-Soluble Phosphates in the Cerebellum and Cerebrum.

Cerebrum.												
Lactate*		Pyruvate		Inorg. P		Phospho- creatine		Adenosine Tri-PO <sub>4</sub>		Adenosine Di-PO <sub>4</sub>		Remarks
Cbt	Cx	Cb	Cx	Cb	Cx	Cb	Cx	Cb	Cx	Cb	Cx	
Controls.												
1.37	1.36	.069	.077	6.72	5.11	1.58	1.49	0.81	0.48	2.56	2.70	
1.50	1.40	.120	.136	4.70	4.93	2.10	1.95	1.30	0.90	1.85	2.06	
1.43	1.38	.095	.106	5.71	5.02	1.84	1.72	1.06	0.69	2.20	2.38	Avg
Nitrogen Mustards.†												
1.80	4.30	.177	.213	4.17	4.24	2.10	1.20	0.39	1.69	3.15	1.32	C <sub>3</sub> H <sub>7</sub> N(C <sub>2</sub> H <sub>4</sub> Cl) <sub>2</sub>
3.48	5.86	.193	.268	3.84	3.60	2.28	1.91	1.43	0.85	1.82	2.51	H <sub>2</sub> C=CHN(C <sub>2</sub> H <sub>4</sub> Cl) <sub>2</sub>
1.66	5.64	.134	.208	4.56	4.20	2.29	1.92	1.13	2.25	2.19	1.00	C <sub>6</sub> H <sub>5</sub> CH <sub>2</sub> N(C <sub>2</sub> H <sub>4</sub> Cl) <sub>2</sub>
1.18	2.76	—	—	4.08	4.56	2.77	1.69	1.71	1.49	1.73	1.57	OC <sub>4</sub> H <sub>8</sub> NC <sub>2</sub> H <sub>4</sub> Cl
3.45	4.08	.277	—	5.19	3.58	1.38	2.30	1.58	1.46	1.97	1.60	C <sub>4</sub> H <sub>9</sub> N(C <sub>2</sub> H <sub>4</sub> Cl) <sub>2</sub>
1.86	2.10	.132	.146	5.94	4.56	3.08	2.16	1.59	0.79	2.00	2.10	C <sub>3</sub> H <sub>7</sub> N(C <sub>2</sub> H <sub>4</sub> Cl) <sub>2</sub>
3.82	7.60	.250	.280	3.72	3.48	3.13	2.03	—	1.36	3.15	1.77	(C <sub>2</sub> H <sub>4</sub> Cl) <sub>2</sub> NCH <sub>2</sub> CH <sub>2</sub> N(C <sub>2</sub> H <sub>4</sub> Cl) <sub>2</sub>
2.46	4.62	.194	.227	4.50	4.03	2.43	1.89	1.30	1.41	2.30	1.70	Avg
Metrazol.												
1.37	2.42	.087	.100	5.18	4.83	2.52	1.50	0.38	0.52	3.48	2.73	
1.50	2.66	.104	.110	4.97	4.68	1.87	1.20	0.53	0.71	2.84	2.35	
1.43	2.54	.095	.105	5.07	4.53	2.19	1.35	0.45	0.62	3.16	2.54	Avg
Aminophylline.												
2.76	9.72	.116	.145	5.88	6.54	1.52	0.42	0.42	0.58	3.00	2.96	16' convulsion
1.56	6.06	.154	.293	(8.21	5.88)	—	—	0.75	1.10	3.11	2.18	13' "
4.34	4.08	.126	.156	5.90	5.64	0.82	0.42	0.11	0.40	3.02	2.70	8' "
3.96	9.72	.112	.106	5.94	6.74	0.60	0.08	0.33	0.56	2.90	2.74	4' "
3.16	7.40	.127	.175	6.48	6.20	0.99	0.31	0.40	0.66	3.01	2.64	Avg
Various.												
2.64	3.51	.240	.130	4.92	3.96	2.05	1.81	1.42	2.28	2.19	0.97	Castorix
1.62	3.48	.126	.164	(6.48	5.62)	§	—	1.62	1.52	1.85	1.78	Dibenamine
2.68	4.50	.188	.254	(6.72	5.36)	—	—	1.16	1.64	2.24	1.42	Br. Camphor
5.74	6.04	.259	.339	(6.12	5.02)	—	—	—	—	—	—	Coramine
2.74	3.60	.164	.206	(7.10	5.18)	—	—	1.44	1.16	2.18	1.74	Strychnine
2.20	4.08	.115	.156	3.90	4.14	2.46	2.22	0.92	0.80	1.86	2.30	Electric shock

\* All values mM/1000 g (wet wt).

† "Cb" and "Cx" designate cerebellar and cerebral cortex, respectively.

‡ Obtained through the courtesy of the University of Chicago Toxicity Laboratory.

§ Pairs of figures enclosed in parentheses are the sum of inorganic phosphate and phosphocreatine for cerebellum and cerebrum, respectively.

|| 2-Chloro 4-dimethyl 1,6-methyl pyrimidine.

ferences were found. The lactic acid level rose only about half as much and the phosphocreatine level was appreciably higher in the cerebellum as compared to the cerebrum. There were no systematic differences discernible in the inorganic phosphorus and ATP-ADP levels; however, this may be due to the fact that many of the seizures were potentiated with carbon dioxide<sup>4</sup> a procedure which tends to minimize changes in the assayed constituents during seizures.<sup>5</sup> These results ob-

tained despite the fact that seizure activity was as violent in the cerebellum as in the cerebrum as judged from the EEG recordings.

*Discussion.* The results reported above point to a quantitative if not qualitative difference in the metabolism of the cerebellum and the cerebrum under the stress of seizures. It is not surprising that this should be so since there are marked histological differences between the two parts. Furthermore, the various parts of the central nervous system

feeding has been shown to increase the incidence of death following roentgen irradiation in mice,<sup>8</sup> experiments were carried out to determine whether or not a cold environment would produce the same effect.

Male mice of N.I.H. stock were received at weaning, divided into treatment groups in which each litter was represented as equally as possible, and placed on a diet of Purina pellets. Three weeks later they were irradiated in groups of 10 made up of approximately equal numbers from each treatment group. The experiments were terminated 4 weeks after irradiation, 98.7% of the observed deaths having occurred between 4 days and 3 weeks.

From one experiment to another we have been unable to duplicate mortality for a given radiation dose, due possibly to genetic heterogeneity of the stock, possibly to fluctuations in X-ray output. Within an experiment these variations should have been largely neutralized by the equal distribution of litters and treatments into radiation groups. Only relative mortality figures are comparable between experiments, and only balanced experiments can be summed.

Irradiation factors were: 170 Kv, 20 ma., added filtration 0.55 mm of aluminum and 0.25 mm of copper, focal distance 50 cm, and dose rate 56 r to 64 r per minute. Total doses were 500 r in the first experiments, 470 r in the last 2 experiments. Cold and hot rooms, thermostatically controlled, were maintained between the limits 9 to 11°C and 29 to 30°C. The 30° temperature was chosen because, according to Harrington,<sup>9</sup> heat production is minimal at an environmental temperature of 30° to 33°C.

Paraffin sections of formalin-fixed tissues were stained with azure eosinate.<sup>9</sup> Frozen sections were stained for fat with oil red O by the method of Lillie and Ashburn.<sup>10</sup> With this method little or no fat is extracted from

the tissue by the staining solution.

In the first 6 experiments a total of 200 mice kept at 30° and 256 at 10°, irradiated with 500 r, were caged together in groups of 10 on sawdust. At each temperature approximately half of the mice were acclimatized for 2 weeks prior to irradiation and half were placed at the temperature immediately after irradiation, where they remained throughout the observation period. In the 30° environment 46% of those acclimatized died compared with 63% of those not acclimatized. At 10°, 52% of those acclimatized died compared with 71% of those not acclimatized. The  $\chi^2$  values of the totals do not indicate significant differences between the temperature groups (2.7 for acclimatized and 1.8 for non-acclimatized mice). Acclimatization at either temperature favored survival  $\chi^2 = 5.4$  for 30°, 8.3 for 10°). The results of the individual experiments were very erratic, however, and thus afford little confidence in the  $\chi^2$  values of the totals.

In this group of experiments survivors at either temperature gained weight during the last 2 weeks of observation. Leucocyte counts made on the first and fourth days after irradiation showed no difference attributable to temperature. Of the non-irradiated controls kept at these temperatures 2 out of 79 at 10° and 1 out of 50 at 30° died from undetermined causes, while the others made satisfactory gains in weight.

Histopathologic changes in the bone marrow, spleen, lymph nodes, thymus, and testis were essentially similar to those recorded in the literature,<sup>11,12</sup> often varied markedly among mice receiving the same treatment, and the severity of the changes did not consistently favor any one treatment group.

In the last 3 experiments the mice were caged singly in suspended mesh cages without sawdust from the time of irradiation (Table I). Ten non-irradiated controls so caged in the 10° environment survived the observation period of 4 weeks without apparent ill ef-

<sup>8</sup> Blount, H. C., Jr., and Smith, W. W., *Science*, 1949, 109, 83.

<sup>9</sup> Lillie, R. D., *Histopathologic Technique*, 1949, Blakiston Co., Philadelphia, Pa.

<sup>10</sup> Lillie, R. D., and Ashburn, L. L., *Arch. Pathol.*, 1943, 36, 432.

<sup>11</sup> Brecher, G., Endicott, K. M., Gump, H., and Brawner, H. P., *Blood*, 1948, 3, 1259.

<sup>12</sup> Henshaw, P. S., *J. Nat. Cancer Inst.*, 1944, 4, 485.

TABLE I.  
Lactate, Pyruvate, and Acid-Soluble Phosphates in Monkey Brain.

Animal No.	Lactate		Pyruvate		Inorg. P		Phospho-creatine		Adenosine Tri-PO <sub>4</sub>		Adenosine Di-PO <sub>4</sub>		Treatment
	Cb*	Cx	Cb	Cx	Cb	Cx	Cb	Cx	Cb	Cx	Cb	Cx	
1	1.68†	1.80	0.171	0.157	3.65	3.93	1.70	1.33	1.40	1.68	1.74	1.27	Control
2	1.44	1.41	0.078	0.083	4.20	4.71	1.27	1.15	1.51	1.43	1.76	1.91	30% CO <sub>2</sub> , 70% O <sub>2</sub> for 3 min.
3	5.22	5.40	0.263	0.269	6.32	5.67	0.47	1.12	1.14	1.18	1.73	1.65	10 sec. electric shock 55 sec. seizure
4	1.74	1.95	0.163	0.168	4.44	4.62	0.62	0.54	1.09	1.34	1.33	1.11	30% CO <sub>2</sub> , 70% O <sub>2</sub> for 3 min. 10 sec. electric shock 55 sec. lag period No seizure

\* "Cb" and "Cx" designate cerebellar and cerebral cortex, respectively.

† All values expressed as mM/1000 g (wet wt) tissue.

compound, the changes are too small, considering the precision of the methods of analysis, to allow conclusions to be drawn without more extensive studies on a larger series of animals. Such a study on monkeys is, at present, beyond our means.

**Summary.** The brains of normal monkeys and those treated with carbon dioxide and

electric shock were fixed with liquid air and analysed for lactate, pyruvate, and acid-soluble phosphates. The levels of these constituents and their change under the above conditions were shown to be of the same magnitude and in the same direction as those found in similarly treated dogs, cats, and rats.

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### 17237. Effect of Environmental Temperature on the Response of Mice to Whole-Body Roentgen Radiation.

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A low environmental temperature for frogs and new-born rats is accompanied by decreased body temperature and oxidative metabolism, and relatively low radiosensitivity.<sup>1-3</sup> In the mouse, when the temperature

regulating mechanism is functioning normally, a cold environment produces only a slight depression of body temperature, and oxygen consumption is elevated.<sup>4,5</sup> This increase in oxidative metabolism has been attributed to increased thyroid activity.<sup>6,7</sup> Since thyroid

1 Patt, H. M., Swift, M. N., and Tyree, E. B., *Fed. Proc.*, 1948, 7, 90.

2 Patt, H. M., Swift, M. N., and Tyree, E. B., *Quart. Rep.*, May-Aug., 1947, A. M. Brues, Editor, AECD 2024, 57.

3 Evans, T. C., Goodrich, J. P., and Slaughter, J. C., *Proc. Soc. Exp. Biol. and Med.*, 1941, 47, 434.

4 Turner, M. L., *Am. J. Physiol.*, 1948, 152, 197.

5 Harrington, L. P., *Am. J. Physiol.*, 1940, 129, 123.

6 Hurst, V., and Turner, C. W., *Am. J. Physiol.*, 1947, 150, 686.

7 Dempsey, R. W., and Astwood, E. B., *Endocrinology*, 1943, 32, 509.

cold environment is to lower the resistance of mice to the lethal action of X-rays.

Subjection to a 10° or 30°C environment for 2 weeks prior to irradiation favors survival of irradiated mice maintained in those environments.

Mice kept in a 30° environment that die following irradiation frequently show fatty changes in the liver.

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### 17238. Effect of Nitrocompounds on Viruses of the Psittacosis-Lymphogranuloma Group.

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*From the Department of Bacteriology and Immunology, Harvard Medical School, Boston, Mass.*

Because of the observation that nitro-acridines showed a marked inhibition of agents of the psittacosis-lymphogranuloma group<sup>1,2</sup> while chloro-substituted and certain other acridines were ineffective,<sup>1</sup> it seemed desirable to investigate the activity of nitro-compounds other than acridines. Substances containing a benzene or furan ring were selected for investigation because certain structural features in nitroacridine are also present in the latter, simpler compounds. While these studies were in progress other publications have described activity of two compounds containing a nitro-phenyl group against viruses of the psittacosis group or rickettsiae. One of these is chloramphenicol (chloromycetin)<sup>3,4</sup> and the other a nitro analogue of D.D.T., 1,1, 1-trichloro-2, 2-bis (para-nitrophenyl) ethane which was found to show some effectiveness against murine typhus in mice.<sup>5</sup>

*Material and methods.* The source of the strains of the viruses of lymphogranuloma

venereum, meningopneumonitis, cat pneumonitis, and mouse pneumonitis is given in a previous publication.<sup>1</sup> Experiments in mice 16 to 18 grams in weight were done with mouse lung passage of these viruses inoculated by the intranasal route. Titrations were done and the infecting dose adjusted so that control mice in each experiment when killed on the 6th day had pulmonary lesions giving a score between 30 and 60.<sup>†</sup> Drugs were given by the intraperitoneal route as single daily doses starting 2 hours after the virus. Control mice received saline intraperitoneally.

The experiments in chick embryos were done with yolk sac passages of the four viruses inoculated either into the yolk sac or the allantoic sac. The technic of the experiments using inoculation into the yolk sac with about 10 LD<sub>50</sub> was identical with that previously described.<sup>1</sup> In the other series of experiments 10 to 100 ID<sub>50</sub> (50% infectious doses) were inoculated into the allantoic sac of 11-day-old chick embryos and a single dose of drug inoculated after the virus either into the allantoic sac or into the yolk sac. Control embryos received saline. The embryos were sacrificed on the 5th day after inoculation and the degree of viral multiplication in the allantoic sac determined by inoculating allantoic fluids from individual treated and control eggs into mice by the intranasal route. Fluids

\* Fellow of the American Bureau for Medical Aid to China, Inc.

<sup>1</sup> Eaton, M. D., vanAllen, A., and Weiner, A., *Proc. Soc. Exp. Biol. and Med.*, 1947, **66**, 141.

<sup>2</sup> Hurst, E. W., *Brit. J. Pharm. and Chemotherapy*, 1948, **3**, 181.

<sup>3</sup> Smadel, J. E., and Jackson, E. B., *Science*, 1947, **106**, 418.

<sup>4</sup> Bartz, Q., Paper presented at Second National Symposium on Recent Advances in Antibiotic Research; also *J. Am. Chem. Soc.*, 1949, in press.

<sup>5</sup> Fitzpatrick, F. K., *Proc. Soc. Exp. Biol. and Med.*, 1949, **70**, 90.

<sup>†</sup> A lesion score of 100 represents death with complete pulmonary consolidation. In surviving mice 80 represents on the average 4+ consolidation; 60, 3+; 40, 2+; and 20, 1+.

TABLE I.  
Incidence of Death Following Irradiation of Mice Caged Singly at 10° and 30°C.

Exp. No.	Dose r	30°C				10°C				$\chi^2$ corrected			
		Acclimatized		Not accl.		Acclimatized		Not accl.		Acclimatization		Temperature	
		Dead	Alive	Dead	Alive	Dead	Alive	Dead	Alive	at 30°	at 10°	Accl.	Not accl.
7	500	8 40%	12	16 80%	4	16	4	20 100%	0	5.10	2.50*	5.10	2.50*
8	470	—	—	16 27%	44	—	—	41 68%	19	—	—	—	19.24
9	470	—	—	23 38%	37	—	—	36 60%	24	—	—	—	4.80
Totals				55 39.3%	85			97 69.3%	43				24.18

\* These values are inexact because 100% of the non-acclimatized mice at 10° died.

fects. Rectal temperatures were determined by an oral clinical thermometer with the bulb completely inserted into the rectum while the mouse was restrained by a wire jacket. Mice at the end of 4 weeks in the 30° environment had an average temperature of 38.4°C, 1.2° higher than those in the 10° environment.

In these experiments, where the mice were unable to huddle together, 69% in the 10° room died compared with 39% in the 30° room. The  $\chi^2$  values for individual experiments are relatively homogeneous, and the value 24.18 for the totals indicates a high degree of probability that the results were not due to chance alone. In the one experiment where acclimatization effect was studied acclimatization again appeared to favor survival.

The detrimental effect of the cold environment was clearly demonstrable when the mice were not allowed to huddle together. The conclusion that resistance to lethal X-ray effects is lowered in a cold environment, when the oxidative metabolism of mice is elevated, is in harmony with results from feeding desiccated thyroid. There are, however, other physiological variables, such as the distribution of blood between peripheral and visceral circulation, which may or may not alter the resistance of the organism to irradiation.

In gross examination of the mice that died following irradiation a uniformly pale or mottled liver was frequently observed in those from the 30° environment. Where 10 mice

were caged together, 48 out of 64 at 30° showed this condition in contrast to 7 out of 76 mice at 10°. Among the mice caged singly, 31 out of 41 had pale or mottled livers at death in contrast to 6 out of 107 mice of the 10° groups. There was no apparent difference in this respect between acclimatized and non-acclimatized mice. The liver was examined microscopically in mice caged singly that died 7 to 14 days after irradiation. From 27 such mice at 30°, 19 showed marked and 4 showed moderate fatty changes, while from 30 such mice at 10° no livers showed marked and 4 showed moderate changes. Among control mice caged 10 together only one out of the 10 kept 2 weeks at 30° showed moderate fatty changes, and none at 10° showed such changes.

The higher incidence of pale, fatty livers in mice that died in the 30° environment was not obviously dependent upon the occurrence of hemorrhage as grossly observed, or upon differences in hemoglobin concentration as determined on 22 mice 11 days after irradiation (at 30°, mean hemoglobin 10.3 g per 100 ml; at 10°, 10.5 g per 100 ml). Since the change occurred chiefly in the groups having the lower incidence of death it is apparently not associated with low resistance to irradiation. The many non-specific factors that could conceivably be responsible for the fatty changes do not permit the conclusion that irradiation had more than an indirect effect in its induction.

*Summary.* The net effect of a sufficiently

cold environment is to lower the resistance of mice to the lethal action of X-rays.

Subjection to a 10° or 30°C environment for 2 weeks prior to irradiation favors survival of irradiated mice maintained in those environments.

Mice kept in a 30° environment that die following irradiation frequently show fatty changes in the liver.

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### 17238. Effect of Nitrocompounds on Viruses of the Psittacosis-Lymphogranuloma Group.

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Because of the observation that nitroacridines showed a marked inhibition of agents of the psittacosis-lymphogranuloma group<sup>1,2</sup> while chloro-substituted and certain other acridines were ineffective,<sup>1</sup> it seemed desirable to investigate the activity of nitrocompounds other than acridines. Substances containing a benzene or furan ring were selected for investigation because certain structural features in nitroacridine are also present in the latter, simpler compounds. While these studies were in progress other publications have described activity of two compounds containing a nitro-phenyl group against viruses of the psittacosis group or rickettsiae. One of these is chloramphenicol (chloromycetin)<sup>3,4</sup> and the other a nitro analogue of D.D.T., 1,1, 1-trichloro-2, 2-bis (para-nitrophenyl) ethane which was found to show some effectiveness against murine typhus in mice.<sup>5</sup>

**Material and methods.** The source of the strains of the viruses of lymphogranuloma

venereum, meningopneumonitis, cat pneumonitis, and mouse pneumonitis is given in a previous publication.<sup>1</sup> Experiments in mice 16 to 18 grams in weight were done with mouse lung passage of these viruses inoculated by the intranasal route. Titrations were done and the infecting dose adjusted so that control mice in each experiment when killed on the 6th day had pulmonary lesions giving a score between 30 and 60.<sup>†</sup> Drugs were given by the intraperitoneal route as single daily doses starting 2 hours after the virus. Control mice received saline intraperitoneally.

The experiments in chick embryos were done with yolk sac passages of the four viruses inoculated either into the yolk sac or the allantoic sac. The technic of the experiments using inoculation into the yolk sac with about 10 LD<sub>50</sub> was identical with that previously described.<sup>1</sup> In the other series of experiments 10 to 100 ID<sub>50</sub> (50% infectious doses) were inoculated into the allantoic sac of 11-day-old chick embryos and a single dose of drug inoculated after the virus either into the allantoic sac or into the yolk sac. Control embryos received saline. The embryos were sacrificed on the 5th day after inoculation and the degree of viral multiplication in the allantoic sac determined by inoculating allantoic fluids from individual treated and control eggs into mice by the intranasal route. Fluids

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<sup>1</sup> Eaton, M. D., vanAllen, A., and Weiner, A., *Proc. Soc. Exp. Biol. and Med.*, 1947, **66**, 141.

<sup>2</sup> Hurst, E. W., *Brit. J. Pharm. and Chemotherap.*, 1948, **3**, 181.

<sup>3</sup> Smadel, J. E., and Jackson, E. B., *Science*, 1947, **106**, 418.

<sup>4</sup> Bartz, Q., Paper presented at Second National Symposium on Recent Advances in Antibiotic Research; also *J. Am. Chem. Soc.*, 1949, in press.

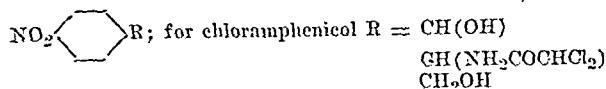
<sup>5</sup> Fitzpatrick, F. K., *Proc. Soc. Exp. Biol. and Med.*, 1949, **70**, 90.

<sup>†</sup> A lesion score of 100 represents death with complete pulmonary consolidation. In surviving mice 80 represents on the average 4+ consolidation; 60, 3+; 40, 2+; and 20, 1+.

TABLE I.  
 Effect of Substituted Nitrobenzenes on Viral Respiratory Infections in Mice.

Virus	Substituent R*	Daily dose, mg	No. of mice	Lesion score		Lung wt (g)	
				T/C†	% change‡	T/C†	% change‡
Cat pneumonitis	COONa	10	43	29/41	-29	0.248/0.288	-14
	CONH <sub>2</sub>	5	18	24/40	-40	0.192/0.247	-23
	C(NH)NH <sub>2</sub>	2	40	30/40	-25	0.284/0.313	-9
	SO <sub>2</sub> NH <sub>2</sub>	4	23	25/52	-52	0.221/0.305	-27
	(Sulfanilamide)	10	14	42/46	-11	0.290/0.293	-1
	Chloramphenicol	2	16	36/38	-5	0.402/0.340	+18
	"	10	16	47/73	-36	0.374/0.460	-19
Mouse pneumonitis	COONa	10	25	39/34	+14	0.295/0.269	+10
	CONH <sub>2</sub>	5	20	35/31	+13	0.220/0.255	-14
	C(NH)NH <sub>2</sub>	2	53	33/38	-13	0.312/0.305	+2
	SO <sub>2</sub> NH <sub>2</sub>	4	21	18/52	-65	0.168/0.260	-35
	(Sulfanilamide)	10	13	20/57	-65	0.173/0.273	-37
	Chloramphenicol	2	16	41/46	-11	0.282/0.376	-24
Lymphogranuloma venereum	COONa	7.5	27	38/39	-3	0.261/0.266	-2
	CONH <sub>2</sub>	5	16	36/40	-10	0.244/0.280	-14
	Chloramphenicol	2	16	23/41	-41	0.204/0.345	-41
Meningo-pneumonitis	COONa	7.5	26	33/35	-6	0.255/0.271	-6
	CONH <sub>2</sub>	5	33	26/32	-19	0.312/0.323	-3
	SO <sub>2</sub> NH <sub>2</sub>	3	31	39/46	-16	0.348/0.379	-8
	Chloramphenicol	2	16	37/51	-28	0.387/0.423	-9
	"	5	16	21/42	-50	0.295/0.456	-35

\* Substituents in para position to nitro group:



† T = Treated mice; C = Control mice.

‡ % change = 100 (-1 + T/C).

were considered to be significantly infected when they produced average lesion scores of 20 or over in the group of 3 mice subinoculated from each embryo.

Drugs were injected by the intraperitoneal route in mice without heating or filtration, insoluble drugs being ground with a small amount of starch and suspended in saline. For inoculation of chick embryos the drugs were sterilized in saline suspension or solution by heating in a boiling water bath for 20 minutes. One nitrofurantoin which was soluble but unstable at 100°C was sterilized by filtration through a fritted glass filter. Dosage up to one-half or one-third of the maximum tolerated dose was used in these experiments.

*Results in mice.* These were expressed as reduction of the observed gross pulmonary consolidation and the weight of the lungs of the treated mice as compared with the controls. Further details on the measurement of

the lung weights and their relation to the degree of consolidation will be found in another publication.<sup>6</sup> In most instances two or three experiments were done with the same drug and when the effects in each experiment were similar the results were averaged. A summary of the findings in mice with compounds containing a nitrophenyl group is presented in Table I. With four compounds, sodium p-nitrobenzoate, p-nitrobenzamide, p-nitrobenzamidine, and p-nitrobenzene sulfonamide, evidence of inhibition of the cat pneumonitis virus was obtained. The most significant effects were those with the two acid amides. With the exception of p-nitrobenzene sulfonamide these substances had no effect against the agent of mouse pneumonitis. Since the latter virus is sensitive to the action of sulfanilamide the positive result with p-

<sup>6</sup> Huang, C., and Eaton, M. D., *J. Bact.*, 1949, in press.

TABLE II.  
Effect of Nitrofurans on Viral Respiratory Infections in Mice.

Virus	Substituent R*	Daily dose, mg	No. of mice	Lesion score		Lung wt (g)	
				T/C	% change	T/C	% change
Cat pneumonitis	$\text{H} \quad \text{O} \quad \text{O}$ $\text{CH}=\text{N}-\text{N}-\text{C}-\text{C}-\text{NH}_2$ $\quad \quad \quad   \quad \quad   $ $\quad \quad \quad \text{H} \quad \quad \text{O}$	3	22	31/48	-35	0.228/0.353	-35
	$\text{CH}=\text{N}-\text{N}-\text{CONH}_2$ $\quad \quad \quad  $ $\quad \quad \quad \text{CH}_2-\text{CH}_2\text{OH}$	2	15	20/45	-56	0.236/0.393	-40
Mouse pneumonitis	$\text{H} \quad \text{O} \quad \text{O}$ $\text{CH}=\text{N}-\text{N}-\text{C}-\text{C}-\text{NH}_2$ $\quad \quad \quad   \quad \quad   $ $\quad \quad \quad \text{H} \quad \quad \text{O}$	3	27	26/56	-54	0.201/0.312	-36
Lymphogranuloma venereum	$\text{H} \quad \text{O} \quad \text{O}$ $\text{C}=\text{N}-\text{N}-\text{C}-\text{C}-\text{NH}_2$ $\quad \quad \quad   \quad \quad   $ $\quad \quad \quad \text{CH}(\text{CO}_2\text{CH}_3)_2$	3	27	6/51	-88	0.178/0.290	-39
	$\text{CH}(\text{CO}_2\text{CH}_3)_2$	2	26	18/37	-51	0.217/0.301	-28
Meningo-pneumonitis	$\text{H} \quad \text{O} \quad \text{O}$ $\text{C}=\text{N}-\text{N}-\text{C}-\text{C}-\text{NH}_2$ $\quad \quad \quad   \quad \quad   $ $\quad \quad \quad \text{H} \quad \quad \text{O}$	$\left\{ \begin{array}{l} 3 \\ 2 \end{array} \right.$	36	11/36	-70	0.211/0.301	-30
	$\text{H} \quad \text{O}$ $\text{C}=\text{N}-\text{N}-\text{C}-\text{C}-\text{NH}_2$ $\quad \quad \quad   \quad \quad   $ $\quad \quad \quad \text{H} \quad \quad \text{O}$	3	31	21/36	-42	0.238/0.340	-30
	$\text{C}=\text{N}-\text{N}-\text{CONH}_2$ $\quad \quad \quad  $ $\quad \quad \quad \text{CH}_2\text{CH}_2\text{OH}$	2	20	26/40	-35	0.288/0.379	-24
	$\text{CH}(\text{CO}_2\text{CH}_3)_2$	2	20	41/49	-16	0.372/0.418	-11


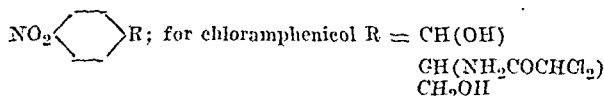
\* All are derivatives of 5-nitro-2-furaldehyde  $\text{NO}_2$   —R where R represents the aldehyde group combined as semioxamazone (I), semicarbazone (III), or diacetate (IV), ethyl-semicarbazone (II), or diacetate (IV).



TABLE I.  
 Effect of Substituted Nitrobenzenes on Viral Respiratory Infections in Mice.

Virus	Substituent R*	Daily dose, mg	No. of mice	Lesion score		Lung wt (g)	
				T/C†	% change‡	T/C†	% change‡
Cat pneumonitis	COONa	10	43	29/41	-29	0.248/0.288	-14
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	SO <sub>2</sub> NH <sub>2</sub>	4	23	25/52	-52	0.221/0.305	-27
	(Sulfanilamide)	10	14	42/46	-11	0.290/0.293	-1
	Chloramphenicol	2	16	36/38	-5	0.402/0.340	+18
	"	10	16	47/73	-36	0.374/0.460	-19
Mouse pneumonitis	COONa	10	25	39/34	+14	0.295/0.269	+10
	CONH <sub>2</sub>	5	20	35/31	+13	0.220/0.255	-14
	C(NH)NH <sub>2</sub>	2	53	33/38	-13	0.312/0.305	+2
	SO <sub>2</sub> NH <sub>2</sub>	4	21	18/52	-65	0.168/0.260	-35
	(Sulfanilamide)	10	13	20/57	-65	0.173/0.273	-37
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	CONH <sub>2</sub>	5	16	36/40	-10	0.244/0.280	-14
	Chloramphenicol	2	16	23/41	-41	0.204/0.345	-41
Meningo-pneumonitis	COONa	7.5	26	33/35	-6	0.255/0.271	-6
	CONH <sub>2</sub>	5	33	26/32	-19	0.312/0.323	-3
	SO <sub>2</sub> NH <sub>2</sub>	3	31	39/46	-16	0.348/0.379	-8
	Chloramphenicol	2	16	37/51	-28	0.387/0.423	-9
	"	5	16	21/42	-50	0.295/0.456	-35

\* Substituents in para position to nitro group:



† T = Treated mice; C = Control mice.

‡ % change = 100 (-1 + T/C).

were considered to be significantly infected when they produced average lesion scores of 20 or over in the group of 3 mice subinoculated from each embryo.

Drugs were injected by the intraperitoneal route in mice without heating or filtration, insoluble drugs being ground with a small amount of starch and suspended in saline. For inoculation of chick embryos the drugs were sterilized in saline suspension or solution by heating in a boiling water bath for 20 minutes. One nitrofurantoin which was soluble but unstable at 100°C was sterilized by filtration through a fritted glass filter. Dosage up to one-half or one-third of the maximum tolerated dose was used in these experiments.

*Results in mice.* These were expressed as reduction of the observed gross pulmonary consolidation and the weight of the lungs of the treated mice as compared with the controls. Further details on the measurement of

the lung weights and their relation to the degree of consolidation will be found in another publication.<sup>6</sup> In most instances two or three experiments were done with the same drug and when the effects in each experiment were similar the results were averaged. A summary of the findings in mice with compounds containing a nitrophenyl group is presented in Table I. With four compounds, sodium p-nitrobenzoate, p-nitrobenzamide, p-nitrobenzimidine, and p-nitrobenzene sulfonamide, evidence of inhibition of the cat pneumonitis virus was obtained. The most significant effects were those with the two acid amides. With the exception of p-nitrobenzene sulfonamide these substances had no effect against the agent of mouse pneumonitis. Since the latter virus is sensitive to the action of sulfanilamide the positive result with p-

<sup>6</sup> Huang, C., and Eaton, M. D., *J. Bact.*, 1949, in press.

TABLE IV.  
Effect of Nitrocompounds on Allantoic Infections with Meningopneumonitis Virus in Chick Embryos.

Name	Dose, mg	Route	Degree of infection as indicated by subinoculation of allantoic fluid into mice			
			LS<20	LS20-50	LS>50	Average L.S.
NF I*	1.0	A	10	12	7	37
Saline		A	2	1	27	83
NF II†	1.0	YS	4	1	1	15
Saline		A	1	2	3	59
Nitroakridin 3582	0.3	A	14	0	0	1.5
Saline		A	1	3	14	74

\* NF I = 5-nitro-2-furaldehyde-semioxamazone autoclaved in dry state.

† Filtered through fritted glass (see Tables II and III for name and formula).

*Results in chick embryos.* Evidence for inhibition of the multiplication of the viruses by nitro-compounds was obtained from experiments in chick embryos. Since the effectiveness of p-nitrobenzoic acid or its amide and p-nitrobenzene sulfonamide seemed to be limited largely to the agent of cat pneumonitis only the experiments in chick embryos with this virus are presented in Table III. Eleven-day-old embryos were infected with a  $10^{-3}$  dilution of yolk sac suspension given by the allantoic route. One hour later the drug was inoculated either by the same route or into the yolk sac. Embryos seldom died as the result of infection but were sacrificed on the 5th day after inoculation and the amount of virus in the allantoic fluid measured by the pulmonary lesion scores resulting from subinoculation intranasally of these fluids into mice which were then sacrificed on the 14th day. As shown in Table III, the allantoic fluids from the majority of the embryos treated with the p-nitrophenyl compounds contained insufficient virus to produce lesion scores of 20 or more in mice while the fluids from many of the control eggs produced lesion scores over 50. The average of the lesion scores from the individual eggs in each group is shown in the last column. The effects were considered significant when the average from the treated eggs was one-half to one-third of the average lesion scores produced by fluids from the control eggs. Chloramphenicol was found to be much more effective against allantoic infections with this virus in chick embryos than it was in mice. The results with sulfanilamide and penicillin are included for comparison. Sulfanilamide

at a dosage of 1 mg, comparable to p-nitrobenzene sulfonamide, had no significant effect. The effect produced by a single dose of 0.3 mg (500 units) of penicillin G was equivalent to or only slightly greater than the effect of the nitro-compounds.

The results of similar experiments with nitrofurans and the agent of meningopneumonitis are presented in Table IV. A number of experiments with these compounds sterilized by heating suspensions in saline or starch-saline at  $100^{\circ}$  for 20 minutes were negative, probably because of heat lability of the drug.¶ Several experiments were then done with the semioxamazone which was sterilized by heating in the dry state, but the results were not as good as would be expected from the experiments in mice. Since 5-nitro-2 furaldehyde-2- $\beta$ -hydroxyethyl semicarbazone is soluble in water to the extent of slightly more than 1 mg per cubic centimeter at room temperature, solutions of this substance were filtered and 1 cc inoculated into the yolk sac. In this experiment a definite inhibition of growth of the meningopneumonitis virus in the allantoic sac was observed. In a similar experiment with the cat pneumonitis virus (see Table III) using a dose of 0.5 mg of this same substance no effect was obtained. The results of an experiment with nitroakridin 3582, (3 nitro- 6,7-dimethoxy-9-(2-hydroxy-3-diethyl amino propylamino) acridine, are also included in Table IV. It is obvious that this compound produced a much more marked inhibition than the nitrofurans in the allantoic sac but from previous investi-

¶ Mary F. Paul, personal communication.

TABLE III.

Effect of Nitro Compounds on Allantoic Infections with Cat Pneumonitis Virus in Chick Embryos.

Name	Dose, mg	Route	Degree of infection as indicated by subinoculation of allantoic fluid into mice†			
			LS<20	LS20-50	LS>50	Average L.S.
Sodium p-nitrobenzoate	10	A	11	6	2	27
p-Nitrobenzamide	10	YS	4	3	1	25
Saline		A	3	9	16	78
p-Nitrobenzene sulfonamide	1	A	6	8	1	22
(Sulfanilamide)	1	A	2	10	3	37
Saline		A	0	10	5	47
NF II‡	0.5	YS	6	3	1	24
Chloramphenicol	1	YS	6	2	0	7
Saline		A	4	6	2	31
(Penicillin G)	0.3	YS	14	3	3	18
Saline		YS	3	3	9	65

\* A = Allantoic; YS = Yolk sac.

† Eggs are divided into three groups; number not significantly infected, lesion score (LS) &lt;20 in mice; number moderately infected, LS20-50; number with heavy infection, LS&gt;50. Average lesion score for entire group of eggs is given in last column.

‡ NF II = 5-nitro-2-furaldehyde-2-β-hydroxyethyl semicarbazone.

nitrobenzene sulfonamide may be attributed to reduction of the nitro group to an amino group in the body. The data from a comparable experiment with sulfanilamide included in Table I show that the effect was very similar in degree. It will be seen, however, that sulfanilamide had little effect on the cat pneumonitis virus while the various nitro-compounds showed slight or moderate activity. With p-nitrobenzamide and p-nitrobenzene sulfonamide, the reduction of pulmonary lesions in mice infected with the agents of lymphogranuloma venereum and meningopneumonitis were very slight and in most cases probably not significant.

Chloramphenicol‡ at a dosage of 2.0 mg/mouse daily had no significant effect against the virus of cat pneumonitis, and only a slight effect against the virus of mouse pneumonitis. At this dosage level a definite inhibitory effect was obtained against the virus of lymphogranuloma venereum but the activity against the agent of meningopneumonitis was of doubtful significance. By increasing the dose definite effects were obtained against the cat pneumonitis virus with 10 mg per day and against the meningopneumonitis virus with 5 mg.

Similar experiments in mice were done with four derivatives of 5-nitro-2 furaldehyde§ using daily doses of 2 to 3 mg per mouse, which was close to the maximum amount tolerated over a period of one week. The mortality from toxic effects of the drugs ranged from 10 to 30% of the treated mice in most of the experiments with these substances. From the results presented in Table II, it will be seen that these nitrofurans had definite inhibitory activity against the agents of mouse pneumonitis, cat pneumonitis, lymphogranuloma venereum, and meningopneumonitis. The most active compound under the conditions of these experiments was the semioxamazone, while the diacetate was found to be the least effective. With the former compound at a dosage of 3 mg marked inhibition of the pulmonary lesions produced by the virus of lymphogranuloma, and the meningopneumonitis virus were observed. When the dosage was reduced to 2 mg toxicity of the drug was not evident in the 6-day period of the experiment, but the effect on the pulmonary lesions produced by the meningopneumonitis virus was not as great.

§ 5-nitrofuraldehyde diacetate was purchased from the Eastman Kodak Co. The other three compounds were given by Dr. Mary F. Paul of the Eaton Laboratories, Inc.

‡ Kindly furnished by Dr. F. Stimpert, Parke Davis & Company.

tributed to reduction of the  $\text{NO}_2$  group to  $\text{NH}_2$  *in vivo*. The possible effect of this reduction in other compounds has not to our knowledge been investigated.

From a consideration of the spectrum of antiviral activity of these substances it is evident that the nitro-compounds as a group, including nitroacridines, have a somewhat similar range of activity while differences from sulfonamides may be found. The agent of mouse pneumonitis which is highly sensitive to sulfonamides, has a relatively low sensitivity to nitroacridine<sup>1</sup> and is not inhibited significantly by derivatives of p-nitrobenzene with the exception of chloramphenicol. The cat pneumonitis and meningo-pneumonitis viruses are resistant to sulfonamides but sensitive to the action of nitroacridines and to most of the other nitro compounds in the case of the former virus but only to nitrofurans and chloramphenicol in the case of meningopneumonitis.

Under the conditions of these experiments the nitrofurans seemed to have a wider range of activity against viral respiratory infections in mice than did the other nitro compounds. The results with chloramphenicol are in agreement with the report of Smadel and Jackson<sup>8</sup> who demonstrated activity of this substance against the agents of lymphogranuloma venereum and psittacosis in chick embryos and against psittacosis in mice inoculated by the intraperitoneal route but were unable to find significant activity against these viruses in mice inoculated by the intracerebral route. From the results presented in Tables I and II it may be seen that chloramphenicol seemed to be less active against the agent of cat pneumonitis than were some of the other nitro compounds. On the other hand, chloramphenicol was more active against the agents of lymphogranuloma venereum and meningopneumonitis than were any of the other nitrophenyl compounds, but

was somewhat less active than the nitrofurans.

While the observations reported here reveal interesting relationships between chemical structure and activity against the viruses of the psittacosis-lymphogranuloma group, it is doubtful that the chemotherapeutic index of any of the substances tested is favorable enough to justify human use. Although sodium p-nitrobenzoate is of low toxicity in mice it has a relatively low activity which is limited almost entirely to the agent of feline pneumonitis. Nitrosulfonamides have been used in human beings for other purposes,<sup>9</sup> but their activity against the psittacosis-lymphogranuloma group is also limited. The nitrofurans have a relatively high degree of activity against these viruses but they have a definite toxic action and have been shown to produce delayed lethal effects in guinea pigs several days after discontinuance of administration.<sup>10</sup> Chloramphenicol is probably among the least toxic of the substances tested but its activity against the agents of the psittacosis-lymphogranuloma group does not seem to be greater than that of penicillin or the sulfonamides.<sup>8</sup>

**Summary.** Several substituted nitrobenzenes including chloramphenicol (chloromycetin) show inhibitory activity against the viruses of meningopneumonitis, lymphogranuloma venereum, cat pneumonitis, and mouse pneumonitis. Several nitrofurans were also shown to inhibit the growth of these viruses. The chemotherapeutic activity of these various substances is compared and the relation of chemical constitution to activity discussed. Although chloramphenicol is probably the least toxic of the substances tested, it did not show striking superiority to other nitro-compounds in activity against viruses of the psittacosis-lymphogranuloma group under the conditions of these experiments.

<sup>9</sup> Major, R. H., *J. Lab. and Clin. Med.*, 1946, **31**, 219.

<sup>10</sup> Wolinsky, E., Wetzel, V., and Steenken, W. J., *Proc. Soc. Exp. Biol. and Med.*, 1949, **70**, 483.

<sup>7</sup> Hamilton, H. L., *Proc. Soc. Exp. Biol. and Med.*, 1945, **59**, 220.

<sup>8</sup> Smadel, J. E., and Jackson, E. B., *Proc. Soc. Exp. Biol. and Med.*, 1948, **67**, 478.

TABLE V.  
Effect of Nitrocompounds on Yolk Sac Infections in Chick Embryos.

Virus	Drug*	Dose	Degree of infection†				Mortality ratio
			LS<20	LS20-50	LS>50	D	
Mouse pneumonitis	PNB	9 mg	2	2	0	5	5/9
	Saline		3	1	1	8	8/13
Cat pneumonitis	PNB	9 mg	8	0	1	2	2/11
	Saline		5	0	0	10	10/15
	NF II	0.5 mg	7	0	3	0	0/10
	Saline		0	0	2	7	7/9
Meningo-pneumonitis	PNBa	10 mg	0	1	1	4	4/6
	Saline		0	0	4	4	4/8
	NF II	0.5 mg	4	2	0	0	0/6
	Saline		0	1	1	9	9/11

\* PNB = p-Nitrobenzoic acid-sodium salt.

PNBa = p-Nitrobenzoic acid-amide.

NF II = 5-nitro-2-furaldehyde-2- $\beta$ -hydroxyethyl semicarbazone sterilized by filtration.

† In eggs surviving to 8 days. D represents number of embryos dead by 8 days.

gations,<sup>1</sup> it seemed to be less effective than the nitrofurans against the meningopneumonitis virus in the lungs of mice.

The results of experiments in which both drug and virus were inoculated into the yolk sacs of chick embryos are presented in Table V. Sodium p-nitrobenzoate in large doses had no effect on the multiplication of the mouse pneumonitis virus but definite evidence of inhibition of the virus of cat pneumonitis was obtained. In one experiment p-nitrobenzamide had no effect on the agent of meningopneumonitis. The compound 5-nitro-2-furaldehyde-2- $\beta$ -hydroxyethyl semicarbazone inhibited the growth of both the cat pneumonitis and meningopneumonitis virus in the yolk sac. While most of the control eggs in these two experiments were either dead or heavily infected by the 8th day after inoculation, the majority of the treated eggs survived with insignificant amounts of virus, although a few had moderate to heavy infections. These results with the three viruses and two types of compound parallel quite closely the observations with mice as reported above.

**Negative results.** The following additional substances containing a nitrophenyl group were screened against the virus of meningopneumonitis in mice and in some cases against other viruses in mice and chick embryos and found to have no effect: p-nitrobenzene sul-

fonanilide, m-nitrobenzene sulfonamide, sodium p-nitrobenzene sulfonate, p-nitroacetanilide, 3-nitro-4-acet-toluide, N(5-nitro-2-furylidene)-1-aminohydantoin.

**Discussion.** These observations and the results reported by other investigators suggest that a certain chemical structure containing a nitro substituent confers on several substances activity against viruses of the psittacosis-lymphogranuloma group. The only chemical grouping common to most of the active nitro compounds so far found is the ring-system of carbon atoms with conjugated double bonds thru which the nitro group is joined to various chemical complexes of rather wide diversity.

It is likely that the nature of the substituent on the benzene ring in the para position to the nitro group plays an important part in determining chemotherapeutic and pharmacologic properties. An analogy with the sulfonamides exists in that the substituent on the sulfonic acid group of the latter compounds increases or decreases their activity. Although some of the compounds listed in the present work resemble p-aminobenzoic acid, others such as the nitrofurans have a structure which is quite different from PABA. Since PABA itself has been found to be inactive against agents of the psittacosis-lymphogranuloma group<sup>6,7</sup> the effects of p-nitrobenzoic acid and its derivatives cannot be at-

tributed to reduction of the  $\text{NO}_2$  group to  $\text{NH}_2$  *in vivo*. The possible effect of this reduction in other compounds has not to our knowledge been investigated.

From a consideration of the spectrum of antiviral activity of these substances it is evident that the nitro-compounds as a group, including nitroacridines, have a somewhat similar range of activity while differences from sulfonamides may be found. The agent of mouse pneumonitis which is highly sensitive to sulfonamides, has a relatively low sensitivity to nitroacridine<sup>1</sup> and is not inhibited significantly by derivatives of p-nitrobenzene with the exception of chloramphenicol. The cat pneumonitis and meningopneumonitis viruses are resistant to sulfonamides but sensitive to the action of nitroacridines and to most of the other nitro compounds in the case of the former virus but only to nitrofurans and chloramphenicol in the case of meningopneumonitis.

Under the conditions of these experiments the nitrofurans seemed to have a wider range of activity against viral respiratory infections in mice than did the other nitro compounds. The results with chloramphenicol are in agreement with the report of Smadel and Jackson<sup>8</sup> who demonstrated activity of this substance against the agents of lymphogranuloma venereum and psittacosis in chick embryos and against psittacosis in mice inoculated by the intraperitoneal route but were unable to find significant activity against these viruses in mice inoculated by the intracerebral route. From the results presented in Tables I and II it may be seen that chloramphenicol seemed to be less active against the agent of cat pneumonitis than were some of the other nitro compounds. On the other hand, chloramphenicol was more active against the agents of lymphogranuloma venereum and meningopneumonitis than were any of the other nitrophenyl compounds, but

was somewhat less active than the nitrofurans.

While the observations reported here reveal interesting relationships between chemical structure and activity against the viruses of the psittacosis-lymphogranuloma group, it is doubtful that the chemotherapeutic index of any of the substances tested is favorable enough to justify human use. Although sodium p-nitrobenzoate is of low toxicity in mice it has a relatively low activity which is limited almost entirely to the agent of feline pneumonitis. Nitrosulfonamides have been used in human beings for other purposes,<sup>9</sup> but their activity against the psittacosis-lymphogranuloma group is also limited. The nitrofurans have a relatively high degree of activity against these viruses but they have a definite toxic action and have been shown to produce delayed lethal effects in guinea pigs several days after discontinuance of administration.<sup>10</sup> Chloramphenicol is probably among the least toxic of the substances tested but its activity against the agents of the psittacosis-lymphogranuloma group does not seem to be greater than that of penicillin or the sulfonamides.<sup>8</sup>

**Summary.** Several substituted nitrobenzenes including chloramphenicol (chloromycetin) show inhibitory activity against the viruses of meningopneumonitis, lymphogranuloma venereum, cat pneumonitis, and mouse pneumonitis. Several nitrofurans were also shown to inhibit the growth of these viruses. The chemotherapeutic activity of these various substances is compared and the relation of chemical constitution to activity discussed. Although chloramphenicol is probably the least toxic of the substances tested, it did not show striking superiority to other nitro-compounds in activity against viruses of the psittacosis-lymphogranuloma group under the conditions of these experiments.

<sup>9</sup> Major, R. H., *J. Lab. and Clin. Med.*, 1946, **31**, 219.

<sup>10</sup> Wolinsky, E., Wetzel, V., and Steenken, W. J., *Proc. Soc. Exp. Biol. and Med.*, 1949, **70**, 483.

<sup>7</sup> Hamilton, H. L., *Proc. Soc. Exp. Biol. and Med.*, 1945, **59**, 220.

<sup>8</sup> Smadel, J. E., and Jackson, E. B., *Proc. Soc. Exp. Biol. and Med.*, 1948, **67**, 478.

TABLE V.  
Effect of Nitrocompounds on Yolk Sac Infections in Chick Embryos.

Virus	Drug*	Dose	Degree of infection†				
			LS<20	LS20-50	LS>50	D	Mortality ratio
Mouse pneumonitis	PNB	9 mg	2	2	0	5	5/9
	Saline		3	1	1	8	8/13
Cat pneumonitis	PNB	9 mg	8	0	1	2	2/11
	Saline		5	0	0	10	10/15
	NF II	0.5 mg	7	0	3	0	0/10
	Saline		0	0	2	7	7/9
Meningo-pneumonitis	PNBa	10 mg	0	1	1	4	4/6
	Saline		0	0	4	4	4/8
	NF II	0.5 mg	4	2	0	0	0/6
	Saline		0	1	1	9	9/11

\* PNB = p-Nitrobenzoic acid-sodium salt.

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**Summary.** Several substituted nitrobenzenes including chloramphenicol (chloromycetin) show inhibitory activity against the viruses of meningopneumonitis, lymphogranuloma venereum, cat pneumonitis, and mouse pneumonitis. Several nitrofurans were also shown to inhibit the growth of these viruses. The chemotherapeutic activity of these various substances is compared and the relation of chemical constitution to activity discussed. Although chloramphenicol is probably the least toxic of the substances tested, it did not show striking superiority to other nitro-compounds in activity against viruses of the psittacosis-lymphogranuloma group under the conditions of these experiments.

<sup>9</sup> Major, R. H., *J. Lab. and Clin. Med.*, 1946, **31**, 219.

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<sup>1</sup> Hamilton, H. L., *Proc. Soc. Exp. Biol. and Med.*, 1945, **59**, 220.

<sup>8</sup> Smadel, J. E., and Jackson, E. B., *Proc. Soc. Exp. Biol. and Med.*, 1948, **67**, 478.



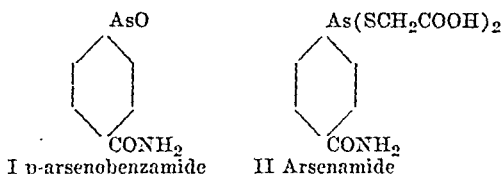
# 17239. Inhibition of Agents of the Psittacosis-Lymphogranuloma Group by P-Arsenobenzamide.

MONROE D. EATON, CHARLOTTE G. LEVENSON, AND CHARLOTTE HANKS.

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Recent investigations of nitro-analogues of para amino benzoic acid have revealed activity of certain of these compounds against agents of the psittacosis-lymphogranuloma group.<sup>1</sup> Because of these observations it seemed of interest to test compounds recently available in which arsenic is substituted for the nitrogen atom and p-arseno benzamide was selected because it may be considered an arsenic analogue of p-nitro benzamide. The latter substance has shown slight inhibitory activity against the virus of feline pneumonitis. p-Arsenobenzamide and the dithioglycollate, known as Arsenamide,\* were tested in mice and chick embryos by the methods de-

The results are summarized in Table II. When the substance was given one hour after inoculation of the virus almost complete inhibition of the growth of the agent of cat pneumonitis was observed, as determined by sub-inoculation of the allantoic fluids of individual eggs into mice. At 24 and 48 hours after inoculation Arsenamide produced partial inhibition of the growth of this virus. The results with meningopneumonitis virus were less striking but partial inhibition of growth in the allantoic sac was obtained when the drug was given one hour after the virus. In mice no significant effect against this virus was obtained (Table I).



scribed in a current publication<sup>1</sup> against the agents of meningopneumonitis, cat pneumonitis and mouse pneumonitis.

The results of experiments on pneumonia in mice are summarized in Table I. In mice inoculated intranasally with the agents of mouse or cat pneumonitis and treated with daily intraperitoneal doses of 0.4 mg of p-arsenobenzamide the gross pulmonary lesions were reduced by about 50 per cent as compared with the controls, and there was also significant reduction in consolidation as measured by the lung weights. The thioglycollate derivative, Arsenamide, was about equally effective against the agent of cat pneumonitis.

Chick embryos infected by the allantoic route were given a single dose of 0.2 mg of Arsenamide by the same route after the virus.

*Comment.* When tested under the same experimental conditions, the activity of p-arsenobenzamide and Arsenamide compares favorably on a weight basis with some of the nitro-compounds including chloromycetin but their toxicity is relatively high and further investigation may show their range of activity to be limited.

As other arsenic compounds have not been examined these preliminary results do not establish any relation of structure to activity against the psittacosis-lymphogranuloma group and further studies will be necessary to assess the significance of the structural relation of this compound to p-aminobenzoic acid and p-nitrobenzamide. Williamson and Lourie<sup>2</sup> have reported that PABA interferes with the trypanocidal action of  $\gamma$  (p-arsenophenyl)-butyric acid, a compound of somewhat similar structure, and Schleyer and Schnitzer<sup>3</sup> find that esters and amides of substituted benzoic acids, but not the acids themselves, antagonize the effect of mapharsen and acriflavine.

*Summary.* p-Arsenobenzamide and its

<sup>1</sup> Eaton, M. D., Huang, C., and Levenson, C. G., *Proc. Soc. Exp. Biol. and Med.*, 1949, **71**, 501.

\* Both supplied by Dr. Marlin T. Leffler of the Abbott Laboratories.

<sup>2</sup> Williamson, J., and Lourie, E. M., *Ann. Trop. Med. and Parasit.*, 1947, **41**, 278.

<sup>3</sup> Schleyer, W. L., and Schnitzer, R. J., *J. Immunol.*, 1948, **60**, 265.

TABLE I.  
Effect of p-Arsenobenzamide and its Dithioglycollate on Viral Respiratory Infections in Mice.

Virus	Compound	Daily dose, mg	No. of mice	Lesion score		Lung weight	
				T/C*	% change†	T/C*	% change†
Cat pn.	I	0.4	27	21/40	-47	0.203/0.311	-35
" "	II	0.5	32	22/39	-44	0.266/0.311	-15
Mouse pn.	I	0.4	27	11/25	-56	0.161/0.209	-23
Meningo-pneum.	I	0.2	16	30/35	-15	0.297/0.325	-8

\* T = Treated mice. C = Control mice.

† % change = 100(-1+T/C).

TABLE II.  
Effect of Arsenamide on Allantoic Infections in Chick Embryos.

Virus	Drug* time After virus, hr	Degree of infection as indicated by subinoculation of allantoic fluids into micet			
		LS<20	LS20-50	LS>50	Av. L.S.
Cat pneumonitis	1	12	0	0	3.0
	Sal.	2	5	7	52.0
	24	7	5	0	16.3
	48	15	1	1	15.2
	Sal.	3	1	4	46.0
Meningo-pneumonitis	1	10	5	2	22.0
	Sal.	0	1	11	87.0

\* Dose 0.2 mg/egg by allantoic route.

† LS<20, allantoic fluid not significantly infected; LS20-50, moderate infection; LS>50, heavily infected allantoic fluid.

dithioglycollate have a definite inhibitory activity against the viruses of mouse pneumonitis and cat pneumonitis in the lungs of mice. The dithioglycollate also inhibits

growth of the agents of meningopneumonitis and cat pneumonitis in the allantoic sac of chick embryos.

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## 17240. Vitamin B<sub>12</sub> Content of Various Organs and Tissues of the Rat.\*

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The glandular tissues of various domestic animals have long been known to be effective in pernicious anemia when administered orally.<sup>1</sup> Beef liver and kidney have proved to be

the richest source of the active principle, kidney, however, possessing only one-half to two-

We are indebted to Merek and Company, Inc., Rahway, N. J., for crystalline vitamins and the crude B<sub>12</sub> concentrate, and to Dr. B. L. Hutchings of the Lederle Laboratories Division, American Cyanamid Company, Pearl River, N. Y., for synthetic folic acid.

<sup>1</sup> Subbarow, Y., Hastings, A. B., and Elkin, M., *Vitamins and Hormones*, 1945, 3, 237.

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TABLE I.  
 Vitamin B<sub>12</sub> Content of Rat Tissues.

Daily supplement	No. of animals per assay group	Series 1 Total avg gain (g) 2 wk	Series 2 Total avg gain (g) 2 wk	Min. B <sub>12</sub> (γ) per g of sample
None	5	48	37	—
1 γ crystalline B <sub>12</sub>	5	73	66	—
1 g kidney	3	65	51	0.04
1 g " (B <sub>12</sub> )*	3	89	87	0.17
1 g liver	5	47	31	†
1 g " (B <sub>12</sub> )	5	75	66	0.08
1 g intestine	5	38	—	†
1 g " (B <sub>12</sub> )	5	62	—	0.04
1 g heart	1	—	36	†
1 g " (B <sub>12</sub> )	1	—	62	0.07
1 g spleen	1	—	32	†
1 g " (B <sub>12</sub> )	1	—	30	†
2.5 g muscle	5	57	43	†
2.5 g " (B <sub>12</sub> )	5	71	66	Trace

\* (B<sub>12</sub>) From rats that received B<sub>12</sub> concentrate.

† No measurable quantity.

thirds the activity of liver. Spleen, brain, heart, and pancreas are effective to a lesser extent. These same organs from the pig and lamb have likewise been used beneficially in the dietary treatment of pernicious anemia.

An assay method has been reported<sup>2</sup> which can be used to measure fairly quantitatively the activity of anti-pernicious anemia preparations, and all subsequent work with the assay has indicated that vitamin B<sub>12</sub> is the active component being determined. The quantitative response obtained with crystalline vitamin B<sub>12</sub> affords a basis for the estimation of the amount of B<sub>12</sub> present in the material tested. Application of this was made in the testing of various beef and pork samples for B<sub>12</sub> content.<sup>3</sup> The work to be reported was undertaken to determine the relative concentration of vitamin B<sub>12</sub> in various organs and tissues of the rat in an effort to locate the main storage site of the vitamin within the body.

**Experimental. Animals.** The rats used as sources of the organ and tissue samples were obtained as weanlings. They were housed in stock colony cages and given food and water

*ad libitum*. A total of 30 rats were used and all received the corn-soybean ration described previously.<sup>4</sup> Fifteen of the animals were given in addition to this diet a vitamin B<sub>12</sub> concentrate (Merck and Co.) which was mixed directly into the basal ration to provide 3 γ of B<sub>12</sub> per 100 g of food. The rats were kept on these diets for 6 weeks before sacrificing.

**Preparation of samples.** The rats were decapitated, bled, and the tissues and organs removed. The liver, kidney, heart, spleen, small intestine, and the femoral muscles were taken. The livers, kidneys, and intestines were weighed, combined with an equal weight of distilled water, and homogenized in a Waring blender. The skeletal muscle was treated in the same manner except that a 3 to 1 dilution was necessary to obtain a smooth homogenate. All samples were stored at ordinary refrigeration temperature.

**Assay.** The assay method used has been described in a previous paper.<sup>2</sup> It consists of placing weanling rats on a basal ration for a 2-week depletion period and following the growth response during another 2-week period when the material to be tested is given. The samples were administered on alternate days in separate food containers. Four ml of the liver and kidney homogenates were given

<sup>2</sup> Register, U. D., Ruegamer, W. R., and Elvehjem, C. A., *J. Biol. Chem.*, 1949, **177**, 129.

<sup>3</sup> Register, U. D., Lewis, U. J., Thompson, H. T., and Elvehjem, C. A., *Proc. Soc. Exp. Biol. and Med.*, 1949, **70**, 167.

<sup>4</sup> Jaffé, W. G., and Elvehjem, C. A., *J. Biol. Chem.*, 1947, **160**, 287.

every other day, thus supplying the animals with an equivalent of 1 g of the sample per day. Because of the higher dilution of the muscle homogenate and its probable lower B<sub>12</sub> content, 15 ml were administered which provided 2.5 g of muscle tissue per day.

The number of rats used for each assay group is recorded in Table I. A smaller number of rats was necessary in the cases of the kidney, spleen and heart because of an insufficient amount of sample. The assay was run in two series, a different group of rats being used as a source of the organs and tissues in each case.

*Results and discussion.* The data on the vitamin B<sub>12</sub> content of all the rat tissues and organs tested are presented in Table I. The values obtained for the heart and spleen are based on results from one assay animal and, therefore, are to be regarded only as indications of the actual.

The greater weight gain in the first series can be explained by the fact that the basal gain, that is, without supplement, was higher throughout all the groups. However, the relative values for B<sub>12</sub> content of the samples in the two individual series were similar.

The data show that in all instances, except possibly with the spleen, the inclusion of the B<sub>12</sub> concentrate in the animal's diet caused an

increase in the quantity of B<sub>12</sub> in the organs and tissues.

The kidneys were found to be the site of greatest B<sub>12</sub> concentration. Also the results indicate that the vitamin is retained in larger quantity for a longer period of time in the kidneys than in the other organs. The liver, heart, and intestine contained no appreciable amount of vitamin B<sub>12</sub> after the animals had been kept on the corn-soybean ration for 6 weeks. However, the increase was very marked in these organs, as with the kidney, upon the addition of the B<sub>12</sub> concentrate to the rat's diet. The skeletal muscle was not only lowest in B<sub>12</sub> but also remained most nearly constant in content.

*Summary.* The kidney, liver, heart, small intestine and femoral muscles of rats raised on a corn-soybean diet were assayed for vitamin B<sub>12</sub> content.

The kidney was found to be the site of greatest B<sub>12</sub> concentration. The liver, heart, small intestine, and muscle contained no appreciable amount of the vitamin after the animals had been kept on the basal ration for 6 weeks. The amount of B<sub>12</sub> increased in the organs and tissues when vitamin B<sub>12</sub> was added to the diet.

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17241. Observations on the Formation of Connective Tissue Fibers.\*

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Some information on the origin and development of collagenous fibers has been obtained from the study of cultured tissues<sup>1-4</sup> and wound healing.<sup>5</sup> In such material fine fibrils can be observed to develop between the cells. These subsequently increase in number and finally coalesce to form larger fibers. It is generally thought that the fibrils are organized from an intercellular protein that is produced by or at least influenced by the surrounding cells, but the precise mechanism is not understood.

One approach to the examination of this problem is by way of direct observations on the early submicroscopic sequences involved in fiber formation. To that end we have utilized the combined methods of electron microscopy and tissue culture.

\* Aided in part by a grant from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council.

<sup>†</sup> Studies made during the tenure of a fellowship in Cancer Research of the American Cancer Society, recommended by the Committee on Growth of the National Research Council.

<sup>1</sup> Maximow, A., *Centr. allg. Path.*, 1929, **43**, 145.

<sup>2</sup> McKinney, R. L., *Arch. exp. Zellforsch.*, 1929, **9**, 14.

<sup>3</sup> Momigliano-Levi, G., *Z. Zellforsch.*, 1932, **16**, 389.

<sup>4</sup> Bloom, W., and Santstrom, R. H., *Anat. Rec.*, 1935, **64**, 1.

<sup>5</sup> Stearns, M. L., *Am. J. Anat.*, 1940, **67**, 55.

The material studied has included fibrous arrays formed *in vitro* in association with explants of chick embryo skin and foregut, rabbit thymus and rat pericardium.<sup>‡</sup> In preparation for microscopy the cultures containing these arrays were washed in a balanced salt solution, fixed briefly over vapors of OsO<sub>4</sub> and thereafter mounted on electron microscope screens by the same technics that are used for cells.<sup>6</sup>

Only areas of the culture in which the plasma clot had been completely lysed were suitable for microscopy. In such areas the presence or absence of connective tissue fibers could be determined with the light microscope. Generally the cell population was sparse. Some units appeared as macrophages and fibrocytes and were spread out quite thinly on the coverglass whereas others of unknown nature remained rounded up and were dispersed over the surface of the fiber mat.

As was more or less expected, it was found that these fibrous mats contain a great many more fibers than are apparent with the light microscope. In fact, the predominant type, hereinafter referred to as unit fibers, has a diameter usually less than 500 Å (Fig. 1 to 5). They are long slender strands which, over the

<sup>‡</sup> All micrographs used for illustrations are of fibers that had developed from explants of chick embryo skin after 9 days in culture.

<sup>6</sup> Porter, K. R., Claude, A., and Fullam, E., *J. Exp. Med.*, 1945, **81**, 233.



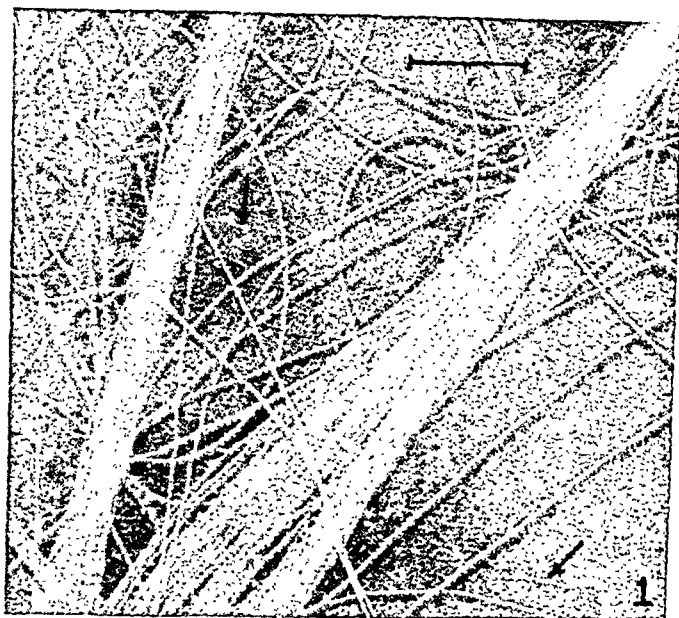


FIG. 1.

Micrograph showing numerous unit fibers and two large collagen-like strands made up of unit fibers. Beaded protofibrils are present in the background and indicated by arrows. Specimen lightly shadowed with gold, 12° angle. Mag. 15,734.

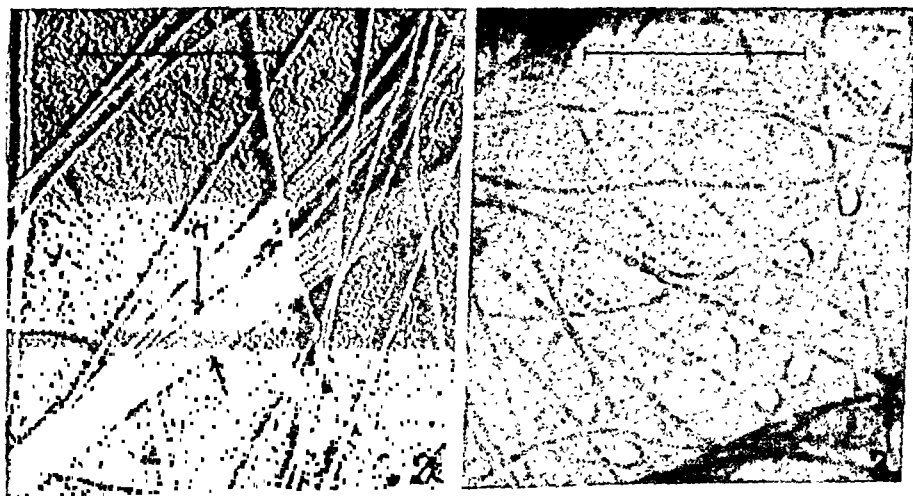


FIG. 2. Micrographs of gold-shadowed preparation showing banded structure of unit fibers (arrows). The periodicity is approximately 270 Å. At (a) there is some evidence of the development of the larger 640 Å period of collagen. Mag. 26,900.

FIG. 3. Micrograph of unit fibers in which the bands or striae are grouped in three's to form the repeating axial pattern of collagen. The intra-period bands are observed to be unequal in size and density. Mag. 30,500.

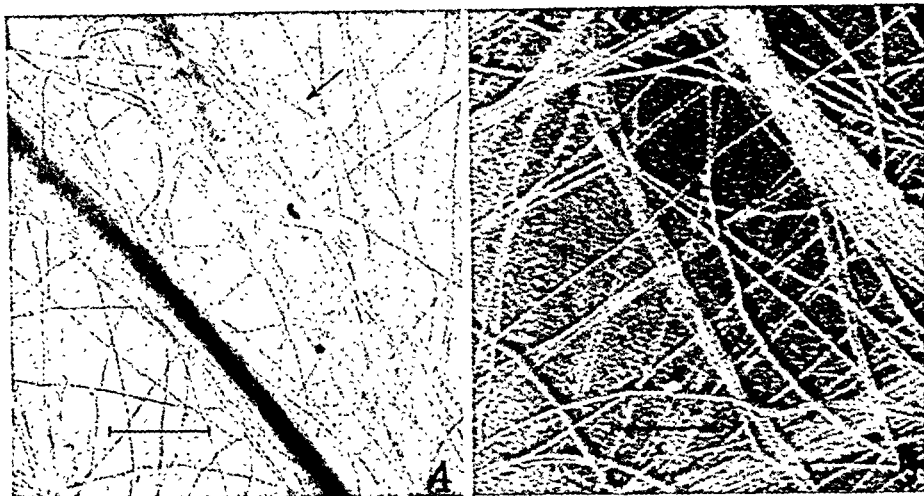


FIG. 4. Micrograph showing large-period component (arrow) of preparations organized as a fiber with densities spaced uniformly at approximately 100 Å. Mag. 13,700.

FIG. 5. Micrograph of similar material (arrow) shadowed with gold, 12° angle. Mag. 14,000.

entire width of a single field of a micrograph (12  $\mu$ ), may not vary appreciably in width. At their ends they taper off gradually into slender threads 50 Å or less in diameter. The unit fibers are all striated or banded, but there is some variation in the organization of the striae or bands. In many fibers, and more especially in those of small diameter, the striae are of uniform size and evenly spaced at approximately 270 Å (Fig. 2). In other unit fibers, which are the larger and presumably more completely formed, the striae are compressed into groups of 3 which have an over-all length of 650 to 800 Å (Fig. 3). When thus grouped, the bands show some differences, 2 appearing larger and denser than the third. The pattern of these bands is repeated in each group or major period along the fiber axis so that not only each period but the whole fiber is polarized (Fig. 2 and 3).

The larger strands of the preparations—those that can be seen with the light microscope—are compound in structure (Fig. 1 to 5). They are composed of varying numbers of unit fibers, and hence, vary in diameter. The size most frequently encountered in these preparations is 0.5  $\mu$  or less. Usually these strands, when not stretched, show the characteristic periodicity of collagen<sup>7,8</sup> (640 Å) with striations extending across a part or all of

the bundle. The typical collagen-like striation of these compound strands shows 2 prominent intra-period striae and one less prominent, in each repeating pattern. The more dense striae correspond to the double elevation apparent in the image of the metal-shadowed, dried fiber.

In part of the preparations where areas of the supporting plastic membrane are exposed, a fine fibrous component is frequently encountered. This consists of very slender filaments or protofibrils, 50 to 100 Å in diameter, which are similar to the ultimate tapered ends of the unit fibers. They are usually beaded. The distance between the bead varies considerably, but not infrequently it is about 270 Å, giving them a periodicity similar to that in small unit fibers. Presumably these protofibrils represent the primary association of the collagen macromolecules.

Neither these fine filaments nor the larger unit fibers have been observed in the material examined to arise as formed structures from cells.

There is a fourth component of these preparations that appears as aggregations of even-

<sup>7</sup> Schmitt, F. O., Hall, C. E., and Jakus, M., *J. Cell. and Comp. Physiol.*, 1942, 20, 11.

<sup>8</sup> Gross, J., and Schmitt, F. O., *J. Exp. Med.*, 1948, 88, 555.

ly-spaced parallel bands or densities. The organization of these is usually in the form of a fiber (Fig. 4 and 5), but occasional patches of irregular outline have been observed. The periodicity displayed measures as a rule between 1000 to 1100 Å. The densities in shadowed preparations appear as prominent elevations and there may be extremely little if any material connecting them. There is no evidence of intra-period banding. In most of the collagen preparations examined, this component has been found scattered rather sparsely among the fibers, but it has also been observed without associated fibers. We are therefore unable to decide whether it is related in any way to collagen formation, but are inclined to the view that it represents a component of the connective tissue ground substance.

The available evidence indicates that the major fibrous components of these preparations, exclusive of the last, are collagen. The periodicity of the large compound fibers is like that of collagen, the fibers resist tryptic digestion, and staining technics have identified collagen fibers in cultures paralleling these used for electron microscopy.

The sequence of events leading to mature

fiber formation, while not directly observable, can be reasonably reconstructed from the character and associations of the various components of any fibrous array. Thus the relation of the unit fibers to the larger strands is clear; they are obviously the component units and appear to have come together into these parallel arrays much the same as unit fibers of fibrin aggregate to produce the larger strands of the formed clot.<sup>9</sup> But the origin of the unit fibers is less evident. They are apparently not spun off the cells. Instead, it is probable that they are formed, as in fibrin, through a lateral association of several protofibrils and a progressive deposition of molecular collagen on their surfaces.<sup>10</sup> Since the more slender unit fibers show a fine even periodicity, it appears that the larger collagen pattern of the mature fiber is a secondary development. These and other phenomena of connective tissue fiber formation will be considered more completely in a later report.

<sup>9</sup> Hawn, C. V. Z., and Porter, K. R., *J. Exp. Med.*, 1947, **86**, 285.

<sup>10</sup> Porter, K. R., and Hawn, C. V. Z., *J. Exp. Med.*, 1949, **90**, 225.

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## 17242. The Cellular Transfer of Cutaneous Hypersensitivity to Tuberculin in Man.

H. SHERWOOD LAWRENCE. (Introduced by William S. Tillett.)

From the Department of Medicine, New York University College of Medicine, and the Third Medical Division of Bellevue Hospital.

It has been demonstrated by Chase<sup>1</sup> that transfer of specific cutaneous hypersensitivity of the "delayed type" to tuberculin can be accomplished in unsensitized guinea pigs by the injection of leucocytes isolated from peritoneal exudates produced in sensitized guinea pigs.

It is the purpose of this report to describe the transfer of cutaneous hypersensitivity to tuberculin in man by the intradermal injection

of viable leucocytes isolated from the peripheral blood of non-tuberculous humans.

**Materials and Methods.** Utilizing the method of Minor and Burnett<sup>2</sup> it has been possible to isolate and concentrate viable leucocytes from human blood by the addition of bovine fibrinogen, Fraction I (Armour), which accelerates the rate of erythrocyte sedimentation leaving a plasma suspension of leucocytes in the supernatant portion.

<sup>1</sup> Chase, M. W., *PROC. SOC. EXP. BIOL. AND MED.*, 1945, **59**, 134.

<sup>2</sup> Minor, A. H., and Burnett, L., *J. Hematology*, 1948, **7**, 799.

1. *Technic of obtaining leucocytes.* Whole venous blood was drawn and placed in sterile potato tubes containing 0.4 ml of a 100 mg % heparin solution for each 10 ml of blood. The tubes were inverted twice and 1.0 ml of sterile Seitz filtered bovine fibrinogen, Fraction I (Armour) solution, (containing approximately 45 mg fibrinogen per ml) was added for each 10 ml of blood and the tubes inverted twice. The heparinized and fibrinogenized blood was then transferred in 10 ml aliquots to conical centrifuge tubes which were placed in a water bath at 37°C for 1 hour. At this time the erythrocytes have become packed in the lower half of the tube and a suspension of leucocytes is contained in the supernatant plasma. The latter was transferred by capillary pipette to specially constructed 10 ml capillary-tip centrifuge tubes (Machlett, No. A 17-210) and centrifuged in an angle centrifuge at 3000 RPM for 1 hour. The cell-free plasma was then decanted and the packed leucocytes (average volume packed wet cells from each 10 ml plasma suspension = 0.025 ml) were resuspended and washed in either 2.0 ml of Tyrodes solution (pH 7.8-8.3) or 2.0 ml of freshly drawn serum obtained from the tuberculin negative recipient of the cells. The cell suspensions were collected from each tube and pooled in one capillary-tip centrifuge tube to a volume of 10 ml and centrifuged again at 3000 RPM for 30 minutes. The supernatant portion was decanted and the washing and centrifugation of the leucocytes was repeated. The supernatant portion of the second washing was decanted, the volume of packed leucocytes read, and then resuspended in either 1.0 ml of Tyrodes solution or 2.0 ml of serum. This suspension of twice washed leucocytes was then injected intradermally into the tuberculin negative recipient.

The interval between the withdrawal of blood and the injection of leucocytes into the recipient was usually not greater than 6 hours. The leucocytes were not refrigerated or stored and rigid sterile technics were observed in the procedure described.

2. *Dosage of leucocytes and vehicle for suspension.* Either 60 ml or 100 ml of venous

blood was obtained from each tuberculin positive donor with normal total peripheral and differential leucocyte count.

The average volume of packed wet leucocytes isolated from 60 ml whole blood was 0.07 ml. The leucocytes were twice washed with 10 ml of Tyrodes solution (pH 7.8-8.3) and resuspended in 1.0 ml of Tyrodes solution for intradermal injection.

The average volume of packed wet leucocytes isolated from 100 ml whole blood was 0.20 ml. The leucocytes were twice washed with 10 ml of serum and resuspended for intradermal injection in 2.0 ml of serum freshly drawn from the tuberculin negative recipient.

3. *Tuberculin Materials Used in Test.* The subjects were tested with Old Tuberculin (O.T.) or Purified Protein Derivative (PPD). The leucocyte donors reacted to either 0.1 ml (1.0 mg) O.T. or to 0.1 ml (0.005 mg) PPD intradermally with +++ to ++++ reactions after 48 hours. The recipients of the leucocytes had no reaction to either O.T. (1.0 mg) or PPD (0.005 mg) intradermally after 48 hours.

4. *Selection of Donors and Recipients.* The group is comprised of 11 tuberculin negative recipients and 5 tuberculin positive donors. The donors and the recipients of the leucocytes were patients hospitalized on the wards of the Third (NYU) Medical Division of Bellevue Hospital or were normal young adults and ranged in age from 21 to 65 years. They were not suffering from any intercurrent infection, were neither cachectic nor myxedematous and had no clinical, laboratory or roentgenographic evidence of active pulmonary or systemic tuberculosis.

5. *Method of Leucocyte Transfer.* Two methods of leucocyte transfer were used.

a) *Prausnitz-Küstner Passive Transfer of Leucocytes.* Using the passive-transfer method of Prausnitz-Küstner, 1.0 ml of a Tyrode suspension of viable leucocytes obtained from a tuberculin positive donor was injected intradermally into the flexor surface of the forearm of the tuberculin negative recipient. After an interval of 18, 24 or 48 hours the reaction at the site of cell transfer was

ly-spaced parallel bands or densities. The organization of these is usually in the form of a fiber (Fig. 4 and 5), but occasional patches of irregular outline have been observed. The periodicity displayed measures as a rule between 1000 to 1100 Å. The densities in shadowed preparations appear as prominent elevations and there may be extremely little if any material connecting them. There is no evidence of intra-period banding. In most of the collagen preparations examined, this component has been found scattered rather sparsely among the fibers, but it has also been observed without associated fibers. We are therefore unable to decide whether it is related in any way to collagen formation, but are inclined to the view that it represents a component of the connective tissue ground substance.

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<sup>9</sup> Hawn, C. V. Z., and Porter, K. R., *J. Exp. Med.*, 1947, **86**, 285.

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It has been demonstrated by Chase<sup>1</sup> that transfer of specific cutaneous hypersensitivity of the "delayed type" to tuberculin can be accomplished in unsensitized guinea pigs by the injection of leucocytes isolated from peritoneal exudates produced in sensitized guinea pigs.

It is the purpose of this report to describe the transfer of cutaneous hypersensitivity to tuberculin in man by the intradermal injection

of viable leucocytes isolated from the peripheral blood of non-tuberculous humans.

*Materials and Methods.* Utilizing the method of Minor and Burnett<sup>2</sup> it has been possible to isolate and concentrate viable leucocytes from human blood by the addition of bovine fibrinogen, Fraction I (Armour), which accelerates the rate of erythrocyte sedimentation leaving a plasma suspension of leucocytes in the supernatant portion.

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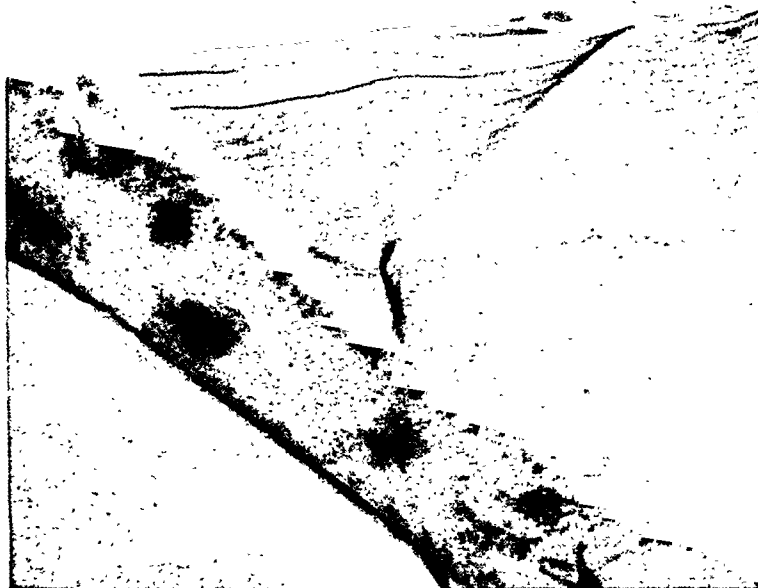


FIG. 1.

Induced Tuberculin Positive Reaction.

Subject S.D. at 24 hr.

Upper: Old cell site challenged with O.T. 1.0 mg.

Middle: New cell site challenged with O.T. 1.0 mg.

Lower: Distant site challenged with O.T. 1.0 mg.



FIG. 2a.

Induced Tuberculin Positive Reaction.

Subject E.K. at 24 hr.

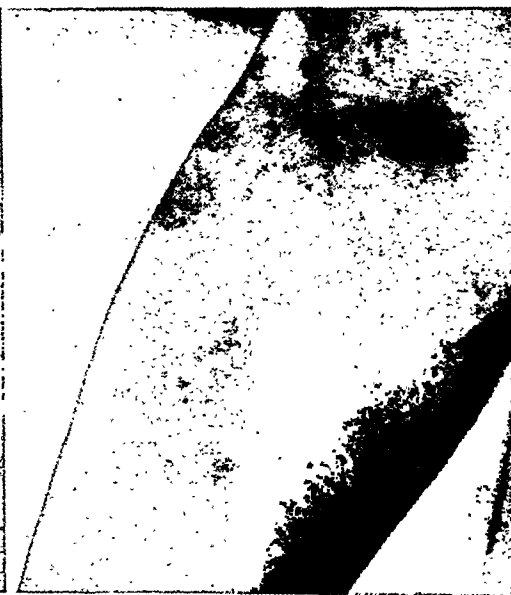
Site distant from cell site challenged with O.T.  
1.0 mg.

FIG. 2b.

Induced Tuberculin Positive Reaction.

Subject E.K. at 24 hr.

Cell site challenged with O.T. 1.0 mg.

TABLE I.  
Cellular Transfer of Cutaneous Tuberculin Hypersensitivity to Tuberculin Negative Recipients, Using the Method of Prausnitz-Küstner.

Tuberculin negative recipient	Tuberculin reaction*† at WBC site—48 hr	Tuberculin reaction distant from WBC site—48 hrs	Duration of induced tuberculin positive state	Tuberculin status of recipient at present
S.D.	++++	+++	>2 mo.	Unknown
E.K.	++++	+++	1 "	Negative
A.G.‡	+++	+++	1 "	"
A.G.‡	Not challenged at WBC site	++	1 wk	"
S.P.	++	+++	2 "	"
N.P.	++	++++	>1 mo.	Unknown

\* Criteria for reading the intradermal tuberculin reaction: The criteria for reading the intradermal tuberculin reaction were the same for all individuals studied:

(a) Tuberculin Negative Reaction: Recorded when no reaction occurred at the intradermal site of P.P.D. 0.1 ml (0.005 mg) or O.T. 0.1 ml (1.0 mg) after 48 hours.

(b) Tuberculin Positive Reaction: Graded according to the severity of the reaction at the intradermal site of P.P.D. 0.1 ml (0.005 mg) or O.T. 0.1 ml (1.0 mg) after 48 hours, as outlined below:

+ —reactions more than 5 mm and not exceeding 10 mm in diameter, showing some redness and definite edema.

++ —reactions more than 10 mm but not exceeding 20 mm in diameter, with an area of redness and edema.

+++ —reactions more than 20 mm but not exceeding 30 mm with marked redness and edema.

++++ —reactions exceeding 30 mm in diameter with marked redness and edema.

† The tuberculin preparation used throughout this series of observations was Old Tuberculin (O.T.).

‡ Same tuberculin negative recipient.

measured and the cell site and a control site some distance from the latter were challenged with 0.1 ml (1.0 mg) O.T. intradermally. Reactions were read in both sites at 15 and 30 minutes and 24, 48, 72 and 96 hours. Three subjects were given additional control intradermal injections of an equal volume of erythrocytes and of serum from the same donor and the sites challenged as above.

b) *Method of Tuberculin Challenge at a site distant from the cell site.* A 2.0 ml serum suspension of leucocytes was injected intradermally in the deltoid area of the tuberculin negative recipient and after an interval of 18 hours, only the skin of the forearm was challenged with 0.1 ml (0.005 mg) PPD intradermally. Readings of the tuberculin reaction in the forearm were made as outlined above.

6. *Control Observations.* Three subjects were given leucocytes from tuberculin negative donors and challenged with tuberculin in the manner described immediately above. These subjects remained tuberculin negative, subsequently were then given leucocytes from tuberculin positive donors.

The first recipient, S.D., became tuberculin positive at the site of leucocyte transfer but not in the control site prepared with donors

serum nor in a site distant from both above sites. After a 16 day interval S.D. was given a second injection of leucocytes from the same tuberculin positive donor and 24 hours later the site of earlier leucocyte transfer, which had faded entirely, the new site of leucocyte transfer and a site distant from both, were each challenged with tuberculin. As is shown in Fig. 1, this recipient developed positive reactions to tuberculin in the old cell site, the new cell site and for the first time in a site distant from the cell sites. This was the first indication that a systemic as well as a local alteration of cutaneous reactivity to tuberculin occurred following leucocytic transfer.

All of the leucocyte recipients who received control intradermal injections of serum and of erythrocytes from tuberculin positive donors, developed positive reactions at the site of leucocyte transfer but not at the sites of serum or of erythrocyte transfer when each site was challenged with O.T. (1.0 mg). The negativity at the sites of serum and erythrocyte transfer persisted until general cutaneous hypersensitivity to tuberculin appeared.

Three recipients (B.H., M.P., R.G.) were injected intradermally with leucocytes obtained from tuberculin negative donors in

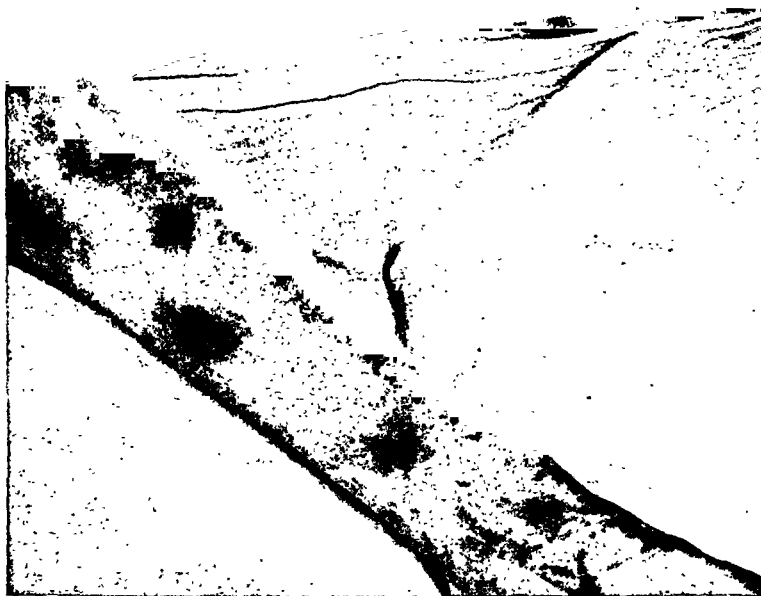


FIG. 1.  
Induced Tuberculin Positive Reaction.  
Subject S.D. at 24 hr.  
Upper: Old cell site challenged with O.T. 1.0 mg.  
Middle: New cell site challenged with O.T. 1.0 mg.  
Lower: Distant site challenged with O.T. 1.0 mg.



FIG. 2a.  
Induced Tuberculin Positive Reaction.  
Subject E.K. at 24 hr.  
Site distant from cell site challenged with O.T.  
1.0 mg.



FIG. 2b.  
Induced Tuberculin Positive Reaction.  
Subject E.K. at 24 hr.  
Cell site challenged with O.T. 1.0 mg.



TABLE II.  
Cellular Transfer of Cutaneous Tuberculin Hypersensitivity to Tuberculin Negative Recipients, Using the Method of Tuberculin Challenge at a Site Distant from the Cell Site.

Tuberculin negative recipient	Tuberculin* status of leucocyte donor	Volume of packed WBC, ml	Tuberculin reaction after WBC transfer at 48 hours	Duration of induced tuberculin positive state	Tuberculin status of recipient at present
B.H.	Negative	0.04	0	4 days	Negative
	Positive	0.05	0		
	"	0.10	+		
M.P.	Negative	0.04	0	3 mo.	"
	Positive	0.05	0		
	"	0.10	++		
R.G.	Negative	0.20	0	Unknown	Positive
	Positive	0.20	++++		
P.O.	"	0.15	+++	>1 mo.	"
S.S.	"	0.20	+++	>1 "	"
A.S.	"	0.20	++	>1 "	"

\* The tuberculin preparation used throughout this series of observations was Purified Protein Derivative (PPD).

order to determine whether the presence of the cutaneous tuberculin hypersensitivity of the leucocyte donor is necessary for the successful transfer of that hypersensitivity to the recipient. In each instance the passive transfer of leucocytes obtained from a tuberculin negative donor had no effect upon the tuberculin reaction of the tuberculin negative recipient. However, the subsequent passive transfer of leucocytes obtained from tuberculin positive donors given to the same three tuberculin negative recipients was followed by the development of cutaneous tuberculin hypersensitivity in each.

It appeared possible but unlikely, that the manipulation or the materials used in the isolation and concentration of the leucocytes, may have directly caused or indirectly contributed to the transfer of cutaneous hypersensitivity to tuberculin. To explore this possibility, leucocytes were obtained from a tuberculin negative donor (M.P.) and the leucocyte suspension injected intradermally into the same tuberculin negative donor (M.P.). This procedure had no effect upon the cutaneous reaction to tuberculin in this individual, whereas the subsequent transfer of leucocytes obtained from a tuberculin positive donor was followed by the development of tuberculin hypersensitivity.

The observations made on subjects (B.H.) and (M.P.) suggest the probable importance of the actual amount or dosage of the packed leucocytes transferred, in determining the development of tuberculin hypersensitivity in the individual recipient. The volume of packed leucocytes obtained from a +++ tuberculin positive donor was 0.1 ml, half of which (0.05 ml) was given to tuberculin negative recipient (B.H.) and half (0.05 ml) given to tuberculin negative recipient (M.P.). Both recipients remained tuberculin negative when subsequently challenged with tuberculin. The transfer of leucocytes obtained from another +++ tuberculin positive donor was repeated, this time 0.1 ml of packed cells was injected into each recipient (B.H. and M.P.). Subsequent challenge with tuberculin resulted in the development of a positive reaction in each recipient.

Of interest is the phenomenon observed in subject P.O. (Fig. 3). This tuberculin negative recipient had no reaction to PPD (0.005 mg) for 72 hours and then 6 hours after the transfer of leucocytes from a tuberculin positive donor, developed a positive reaction at the formerly negative PPD site, which reached its maximum intensity (++) at 48 hours. When challenged with PPD (0.005 mg) 24 hours after leucocyte transfer, this individ-

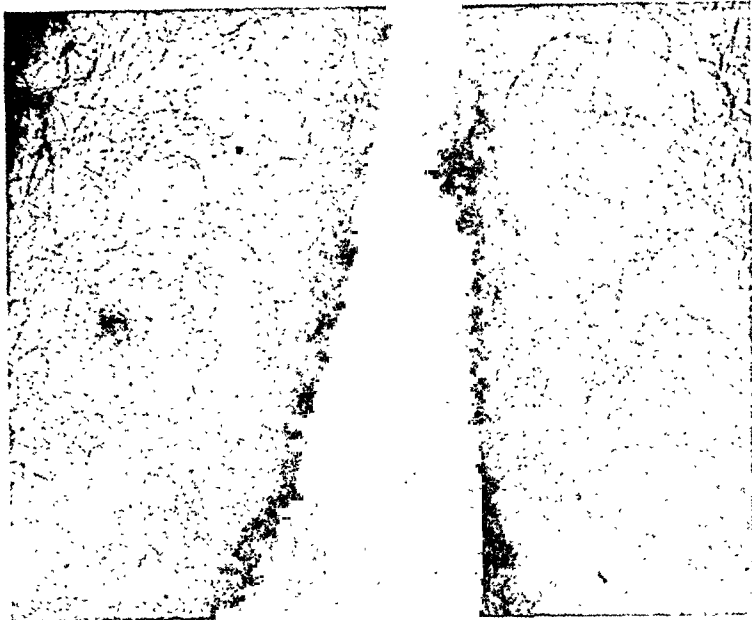


FIG. 3.

Induced Tuberculin Positive Reaction Subject P.O.

Left: Reaction at 48 hours (PPD-0.005 mg) at site distant from cell site, which had been challenged 24 hours after leucocyte transfer.

Right: Reaction at 72 hours (PPD-0.005 mg) at site distant from cell site, which had been negative until 6 hours after leucocyte transfer.

ual developed a positive reaction at the new PPD site which reached its maximum intensity (++++) at 48 hours. The delayed appearance of a positive reaction at the site of a formerly negative tuberculin test has been commented upon by several observers<sup>3</sup> and it usually occurs secondary to the development of a primary tuberculous infection in the interim between testing and the appearance of the positive reaction. There has been no clinical or roentgenographic evidence that a primary tuberculous infection had occurred in subject (P.O.). To substantiate this, is the observation that the degree of his induced cutaneous hypersensitivity to tuberculin has decreased progressively in intensity from a (++++) to a (+) reaction in the month following leucocyte transfer.

The observations tabulated in Table III suggest the probable importance of the degree of tuberculin hypersensitivity of the leucocyte donor, in determining the degree of induced

tuberculin hypersensitivity of the individual recipient.

Of the 6 tuberculin negative recipients who were followed with repeated tuberculin testing after conversion to the tuberculin positive state, 5 spontaneously reverted to tuberculin negativity after a variable period of 4 days to 3 months.

One of the subjects (A.G.) with induced cutaneous hypersensitivity to tuberculin who spontaneously reverted to a tuberculin negative state, was sensitized again with leucocytes obtained from another tuberculin positive donor and subsequently became tuberculin positive for the second time. This subject has since become tuberculin negative again.

The recipients P.O., S.S., A.S. and R.G., with induced cutaneous tuberculin hypersensitivity of more recent origin, are still tuberculin positive at present. However, the degree of tuberculin hypersensitivity of P.O. and S.S. has decreased progressively in intensity from a (++++) to a (+) reaction in the month following leucocyte transfer. The observations

<sup>3</sup> Daniels, M., *Lancet*, 1943, 245, 600.

TABLE III.  
Degree of Induced Tuberculin Hypersensitivity in Relation to Degree of Hypersensitivity of Leucocyte Donor.

Tuberculin positive donor	Degree of tuberculin hypersensitivity of donor	Tuberculin negative recipient per donor	Degree of tuberculin hypersensitivity in recipient following WBC transfer	Duration of tuberculin positive state	Tuberculin status at present
D.M.	+++	S.D.	+++	>2 mo.	Unknown
		E.K.	+++	1 "	Negative
		B.H.	+	4 days	"
		M.P.	++	3 mo.	"
		A.G.†	+++	1 "	"
C.S.	++++	S.P.	+++	2 wk	"
		N.P.	++++	>1 mo.	Unknown
P.B.	++++	A.G.†	++	1 wk	Negative
		P.O.	+++	1 mo.	Positive*
R.H.	++++	S.S.	+++	1 "	" *
		A.S.	++	1 "	" *
		R.G.	++++	Unknown	" *

\* Each recipient still under observation.

† Same tuberculin negative recipient.

made on the recipients with induced cutaneous tuberculin hypersensitivity, who spontaneously reverted to a tuberculin negative state, indicated that the progressive decrease in the intensity of the tuberculin reaction was followed shortly thereafter by the return to tuberculin negativity.

*Discussion.* In twelve consecutive instances the tuberculin negative recipients of viable leucocytes obtained from tuberculin positive donors, subsequently developed cutaneous hypersensitivity to tuberculin. When the leucocytes were obtained from tuberculin negative donors, cutaneous hypersensitivity to tuberculin did not subsequently develop in the tuberculin negative recipient.

The cutaneous hypersensitivity to tuberculin following leucocyte transfer is a transient phenomenon of 4 days to 3 months duration. The induced cutaneous hypersensitivity to tuberculin can be produced again in the same individual.

The degree of induced hypersensitivity to tuberculin seems to bear a relationship to the dosage of leucocytes used and the degree of tuberculin hypersensitivity of the leucocyte

donor.

These observations in man are in agreement with those made by Chase<sup>1</sup> in the guinea pig.

*Summary and conclusions.* 1. It has been possible to passively transfer in 12 consecutive instances cutaneous tuberculin hypersensitivity to tuberculin negative human recipients by means of an intradermal injection of viable leucocytes obtained from the blood of tuberculin positive human donors.

2. The effort at the passive transfer of cutaneous tuberculin hypersensitivity was unsuccessful when the leucocytes used in the transfer were obtained from tuberculin negative donors.

3. The induced cutaneous hypersensitivity to tuberculin is a transient phenomenon of 4 days to 3 months duration, which can be produced again in the same individual.

4. The degree of induced hypersensitivity to tuberculin seems to bear a relationship to the dosage of leucocytes used and the degree of tuberculin hypersensitivity of the leucocyte donor.

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# 17243. Acetylcholine-Like Action of a Product Formed by an Acetylating Enzyme System Derived from Brain.\*

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In 1943 Nachmansohn and Machado extracted from brain an enzyme, choline acetylase, which forms acetylcholine in cell-free solution using the energy of adenosine triphosphate.<sup>1</sup> The acetylation of choline is a complex reaction requiring for full activity in addition to the enzyme system and ATP, the substrates choline and acetate, a co-enzyme,  $K^+$ ,  $Mg^{++}$ ,  $Ca^{++}$  ions and cysteine.<sup>2,3</sup> The choline ester formed enzymatically, assumed to be exclusively acetylcholine, was determined by bioassay with the rectus muscle of the frog. In spite of the intense interest of physiologists in acetylcholine, no adequate chemical methods were available and investigators studying the occurrence or formation of the ester used by necessity bioassays, which are of questionable specificity.

Recently high yields of choline ester were obtained by a purified and concentrated enzyme solution which was prepared from acetone dried powder of rabbit brain by fractional ammonium sulphate precipitation. These high yields made possible the determination of choline ester by a chemical method introduced by Hestrin.<sup>4</sup> The method is based on the reaction of O-acyl groups with hydroxylamine in alkaline medium and may therefore be used for the determination of acetylcholine in presence of choline and acetate.

When tested by bioassay the values of acetylcholine formed showed a striking discrepancy with the figures obtained by chemical determination. Less than half of the total biological activity of the enzymatically formed product could be accounted for by the chemical method. Consequently, the greater part of the effect obtained in the bioassay must be attributed to a product which appears to have the same biological action as acetylcholine but may be distinguished from the latter chemically.<sup>3</sup> The formation of a biologically active product is observed in absence of added choline, whereas under these conditions no acetylcholine formation is observed.

It appeared necessary to ascertain whether the product has acetylcholine-like action in other biological systems besides that observed on the frog rectus. Experiments will be described in this paper in which the action of the enzymatically formed product, henceforth referred to as e.f. product, has been tested on the frog heart and on the blood pressure of cats.

The tests of the effect of the product on blood pressure were performed on cats. The animals were anesthetized with Dial (0.07 - 0.08 g/kilo), injected intraperitoneally. Arterial blood pressure was measured in the carotid artery with a mercury manometer and recorded on smoked paper. The compounds tested were injected into the external jugular vein in a volume of 1 ml.

For the experiments on the frog's heart, bullfrogs (*Rana catesbeiana*) were used. The hearts were removed and perfused with frog's Ringer solution according to the method of Straub, as modified by Kray.<sup>5</sup> Ventricular contractions were recorded with an isotonic lever attached to the apex of the heart.

The enzymatically formed product used in

\* This investigation was supported by research grants from the Office of Naval Research and the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service.

† Rockefeller Foundation Fellow. Present address: Institute of Physiology, University of Chile.

<sup>1</sup> Nachmansohn, D., and Machado, A. L., *Neurophysiol.*, 1943, **6**, 397.

<sup>2</sup> Nachmansohn, D., and Weiss, M. S., *J. Biol. Chem.*, 1948, **172**, 677.

<sup>3</sup> Nachmansohn, D., Hestrin, S., and Voripaieff, H., *J. Biol. Chem.*, 1949, **180**, 249.

<sup>4</sup> Hestrin, S., *J. Biol. Chem.*, in press.

<sup>5</sup> Kray, O., Linstead, R. P., and Todd, D., *J. Pharm. and Exp. Therap.*, 1943, **77**, 113.

TABLE I.

Depressor Effect of the Enzymatically Formed Product on Arterial Blood Pressure of Cat. Bioassay with frog's rectus indicated a content of 30-40  $\mu\text{g}$  ACh equivalents per ml of sample. a and b indicate assays done at different periods of the same experiment.

Exp. No.	Acetylcholine		e. f. product		
	$\mu\text{g}$ ACh	Decrease of blood pressure, %	Dilution of product	Decrease of blood pressure, %	$\mu\text{g}$ ACh equivalents/ml
1	0.25	28	400	26	100
2	0.2	22	400	23	80
	0.5	35	200	35	100
3 a.	0.25	25	400	23	100
	b. 0.5	30	200	28	100
4	0.25	13	400	12	100
	0.5	35	200	29	<100
5 a.	0.2	17	400	14	80
	b. 0.5	26	200	23	100
6 a.	0.2	20	400	24	80
	0.25	36			
	b. 0.5	43	200	35	<100
7	0.2	45	400	42	80

all the experiments was obtained by incubation of the choline acetylating system in a reaction mixture as described recently.<sup>3</sup> No choline was added to the mixture. The blank solutions used as controls were aliquot parts of the reaction mixture which formed on incubation the product. The controls were used at 0 time (without incubation), after the enzyme had been inactivated by short boiling.

*Results. A. Action of product on arterial blood pressure of cats.* Table I summarizes the results obtained in this series of experiments. In all the experiments performed, the e.f. product exerted on arterial blood pressure a depressor action that closely resembles the fall in blood pressure induced by acetylcholine.

The samples used in Experiments 1 to 3

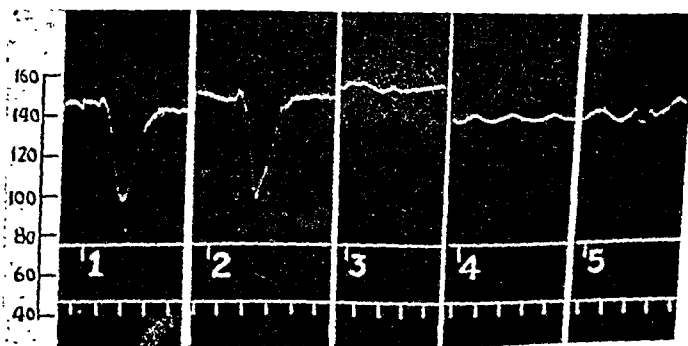


FIG. 1.

Effect of e.f. Product upon Arterial Blood Pressure. Cat.

Upper line, arterial blood pressure; second line, injection marks; third line, time in 10 sec. Scale on the left: mm of Hg. 1. ACh 0.5  $\mu\text{g}$ . 2. e.f. product diluted 1:200. 3. Control diluted 1:50. Between 3 and 4, atropine sulfate (0.4 mg). 4 and 5, as in 1 and 2.

TABLE II.

Effect of the Enzymatically Formed Product on the Amplitude of the Frog Heart Contraction. Bioassay with frog rectus indicated a content of 30-40  $\mu\text{g}$  ACh equivalents per ml of sample.

Exp. No.	Acetylcholine		e. f. product		
	$\mu\text{g}$ ACh	Decrease of amplitude, %	Dilution of product	Decrease of amplitude, %	$\mu\text{g}$ ACh equivalents/ml
1	0.01	60	4000	66	50-60
	0.02	78			
2	0.025	58	1600	48	>40
3	0.05	22	1600	20	80
	0.1	40			
4	0.05	32	2000	32	100
			1600	40	
5	0.05	35	1600	37	80
		40	800	50	
6	0.1	50	800	43	40-60
	0.3	64	400	50	
			200	68	
7	0.025	60	1600	68	50-60
	0.05	82			

were derived from the product of the same experiment, 4 to 7 from another experiment. Compared with bioassay on frog rectus the values obtained were higher, although in the same order of magnitude. The figures in all experiments indicate strikingly good constancy. It may be noted that experiments 6 and 7 were carried out about 2 weeks later. During this interval, however, no loss of activity seems to have occurred.

It was also observed that the depressor action of the e.f. product is regularly suppressed by atropine in doses sufficient to abolish the depressor action of acetylcholine.

Fig. 1 shows a typical experiment. It can be observed that a dose of e.f. product (at 1) and of acetylcholine (at 2) which elicit a marked fall of blood pressure before atropine, becomes completely ineffective once the animal has been atropinized (5 and 6). The injection of a control solution at 3 does not modify the level of blood pressure.

*B. Action of product on the frog's heart.* The results obtained are summarized in Table II. In 7 experiments carried out the e.f. product given in adequate concentrations pro-

duced depression of the activity of the heart, similar to that elicited by the administration of acetylcholine. The depressor action affected primarily the amplitude of the cardiac contractions, the frequency being only slightly decreased if at all.

The figures of Table II show that the concentration of the e.f. product in the samples used is fairly constant, as estimated by their action on the frog's heart. It fluctuates between 40 and 80  $\mu\text{g}$  of acetylcholine equivalents per ml in one sample (1 and 2), between 40 and 100  $\mu\text{g}$  per ml in the second sample used (3 to 7).

The cardiodepressor action of the e.f. product was regularly blocked by atropine in concentrations sufficiently high to abolish the cardiodepressor effects of acetylcholine.

The record in Fig. 2 is typical of this series. At 1 and 2, e.f. product and at 3 acetylcholine, produce a marked decrease in the amplitude of cardiac contractions. At 4, a control solution is used; no effect on cardiac activity is obtained. The heart is then atropinized and at 5 and 6, e.f. product and acetylcholine are applied again as in 2 and 3; the

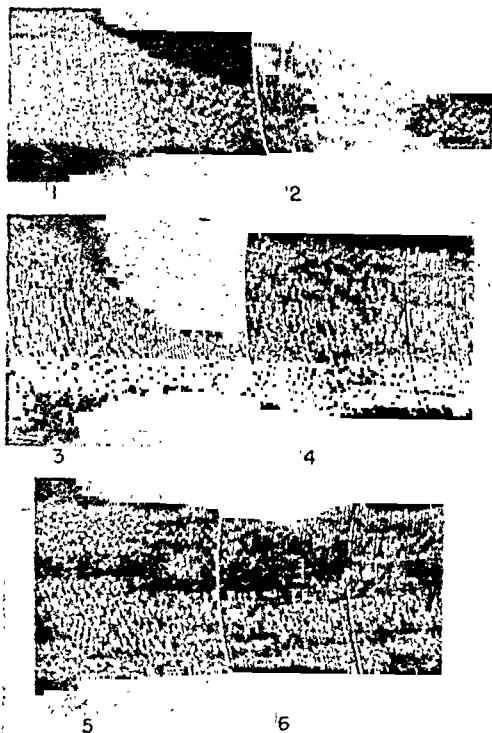


FIG. 2.  
Effect of e.f. Product upon the Ventricular Contractions of the Frog Heart.

1. e.f. product diluted 1:100. 2. e.f. product diluted 1:50. 3. ACh 0.3  $\mu$ g. 4. Control diluted 1:50. 4 and 5. Atropine sulfate 1 mg/liter. 5. ACh as in 3. 6. e.f. product as in 2.

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**Summary.** The pharmacological properties of an enzymatically formed product described recently by Nachmansohn, Hestrin and Voripaieff have been tested. This product, obtained in the choline acetylating system derived from brain extracts, is distinctly different from acetylcholine but has an acetylcholine-like action in the bioassay with frog rectus.

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## 17244. New Substrates for Cholinesterases.

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From the Mayo Foundation, Rochester, Minn.

In previous reports it has been shown that the cholinesterases (ChE) of snake venoms<sup>1-3</sup> and of human erythrocytes<sup>3,4</sup> catalyze the hydrolysis of noncholine esters such as ethyl

chloroacetate and  $\beta$ -chloroethyl acetate. Since both enzymes are typical members of the e-group of cholinesterases,<sup>5</sup> the former assumption that the e-ChE was unable to attack noncholine ester, therefore, should be discarded. Thus, a wide field for the search for new substrates of the e-ChE has been opened.

**Methods.** Esterase activity was measured by the usual manometric procedure.\* In each case tests were also run without sub-

<sup>†</sup> On leave from the University of Basel, Switzerland.

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<sup>3</sup> Zeller, E. A., Fleisher, G. A., and McNaughton, R. A., *Fed. Proc.*, 1949, **8**, 268.

<sup>4</sup> McNaughton, R. A., and Zeller, E. A., *Proc. Soc. Exp. Biol. and Med.*, 1949, **70**, 165.

<sup>5</sup> Zeller, E. A., *Helvet. chim. acta*, 1949, **32**, 94.

\* Twelfth communication: Zeller, E. A., *Helvet. chim. acta*, 1949, **32**, 484.

TABLE I.  
 Enzymatic Hydrolysis of a Mixture of Acetylcholine and Ethoxyethanol Acetate.\*

Substrates	Conc. of substrate	Q	$Q_{\text{ACh}} + Q_{\text{EtA}}$	$Q_{\text{ACh}} + Q_{\text{EtA}}$
ACh	.005 M	5,730	7,490	5,600
EtA	.05 M	1,760		
ACh	.005 M	5,730	8,630	5,080
EtA	.20 M	2,900		
ACh	.01 M	4,700	6,460	4,190
EtA	.05 M	1,760		
ACh	.01 M	4,700	7,600	4,840
EtA	.20 M	2,900		

\* 0.1 ml of purified erythrocyte ChE, 1.0 ml total volume.

strates and without enzymes, and all results recorded in this paper were obtained by subtracting the corresponding blanks. They are expressed in terms of Q, which gives the number of microliters of carbon dioxide evolved from bicarbonate-Ringer per hour per milligram of venom or per milliliter of the enzyme solution.

Sources of e-ChE were dry snake venoms (from the family of the Colubridae) and human erythrocytes. The latter were washed 4 times with saline solution and then hemolyzed with 5 volumes of distilled water. The hemolyzed cells were centrifuged at 8,800 g until a reddish precipitate separated. The supernatant was discarded and the precipitate was resuspended in water; this procedure was repeated 6 to 8 times until the supernatant was clear. The orange-red precipitate contained 50 to 60% of the original ChE activity of the erythrocytes. This preparation will be referred to as "purified erythrocyte ChE."

In the venoms<sup>5</sup> and in the purified erythrocyte ChE preparation no indication of the presence of an "ali"-esterase appeared. The activity toward methyl butyrate, which is found in hemolyzed erythrocytes, completely disappeared after purification. Thus, when both preparations attack an ester, it is highly probable that the e-type of ChE is responsible for this reaction. The sensitivity toward eserine and caffeine<sup>6</sup> and, when possible competition experiments with acetylcholine were used to check this conclusion.

**Results. A. Ethoxyethanol Acetate (EtA).** This glycol derivative is easily attacked by the venoms of *Naia melanoleuca* ( $Q_{\text{EtA}} = 1,720$ ) and *Elaps frontalis* ( $Q_{\text{EtA}} = 170$ ), using a substrate concentration of 0.1 M. Eserine (0.2mM) gives complete inhibition. Purified erythrocyte ChE also catalyzes the hydrolysis of ethoxyethanol acetate. In all cases acetylcholine competed with the non-choline ester, as is seen from the results listed in Table I. The same substance is also hydrolyzed by human serum, a rich source of s-ChE ("pseudo" or "unspecific" ChE). Using a purified erythrocyte ChE and diluted human serum of a similar activity toward acetylcholine ( $Q_{\text{ACh}}$ ), the first enzyme preparation catalyzes the ethoxyethanol acetate much more rapidly than the s-type, in spite of the fact that the affinity between the substrate and the two enzymes is of the same order (compare with Michaelis constant, Fig. 1). Since human serum contains some "ali"-esterase activity<sup>7</sup> which probably is partly responsible for the hydrolysis of the noncholine ester, the ratio might be even more in favor of the e-type.

**B. Desoxycorticosterone Acetate (DOCA).** The extremely low solubility of this substance in water prevented any rapid reaction. The substance was added to the reaction vessels in dry form. Venom from *Naia melanoleuca* ( $Q_{\text{ACh}} = 30,800$ )<sup>5</sup> produced an easily readable hydrolysis ( $Q_{\text{DOCA}} = 8.7$ ). Eserine (0.2 mM) inhibited this reaction 90%. Even purified erythrocyte ChE caused a considerable hy-

<sup>6</sup> Zeller, E. A., and Bissegger, Alfred, *Helvet. chim. acta*, 1943, 26, 1619.

<sup>7</sup> Adams, D. H., and Whittaker, V. P., *Biochem. J.*, 1949, 44, 62.



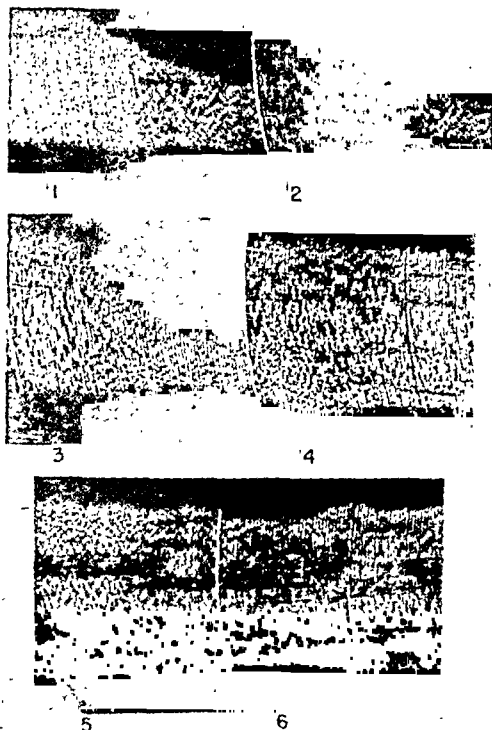


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TABLE II.  
Hydrolysis of Phenyl Acetates in the Presence of Various Cholinesterase Preparations.

Source of ChE	Type	$Q_{AcA}$	Substrate	Q	Q substrate + 0.2 mM eserine
Venom of <i>Bungarus fasciatus</i> †	e	25,000	Phenyl acetate, .002 M	16,200	0
Hemolyzed erythrocytes‡	e	11,750	" " .002 M	7,250	0
Plasma*	s	6,500	" " .002 M	117,000	102,000
Purified erythrocyte ChE§	e	12,000	Acetylsalicylic acid, .06 M	160	0
Venom of <i>Naia melanoleuca</i>	e	30,800	" " .08 M	650	0
Purified erythrocyte ChE¶	e	5,550	p-Nitrophenyl acetate, .003 M	2,680	

Total volume in all cases = 1 ml.

\* 0.002 ml of heparinized human plasma.

† 0.02 mg of dry venom.

‡ 0.1 ml of washed and hemolyzed erythrocytes; washed 3 times with saline solution and hemolyzed with 3 volumes of distilled water. This suspension was diluted with 9 volumes of "Ringer-30."

§ 0.3 ml (cf. "Methods").

|| 1 mg of dry venom.

¶ 0.025 ml.

choline is the physiologic substrate of the e-ChE.

**Summary.** e-Cholinesterase ("true" cholinesterase) from human erythrocytes and snake venoms is capable of catalyzing the hydrolysis of ethoxyethanol acetate, desoxycorticos-

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## 17245. Mobilization of Radioactive Sodium from the Gastrocnemius Muscle of the Dog.\*

PETER W. STONE<sup>1</sup> AND WILLIAM B. MILLER, JR.<sup>2</sup> (Introduced by J. V. Warren.)

From the Whitehead Department of Surgery, Emory University, Atlanta, Ga.

The experimental study reported here was based on the clinical application of intramuscular injection of radioactive sodium 24 in studies of peripheral vascular disease by Elkin *et al.*<sup>1</sup> This technic and the inferences

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drawn from the results of those studies are predicated upon the disappearance of the radioactive sodium by way of the blood stream. However, the possibility existed that a portion of the sodium was being removed by the lymphatic system. A further consideration was that the isotope might diffuse along intramuscular planes, thereby removing itself from the range of the Geiger-Mueller counter, in which event it would erroneously appear that the sodium had been removed by the circulating blood. The study reported here was undertaken, therefore, to determine:

(1) The role of the lymphatics in the mobilization and removal of intramuscularly injected radioactive sodium.

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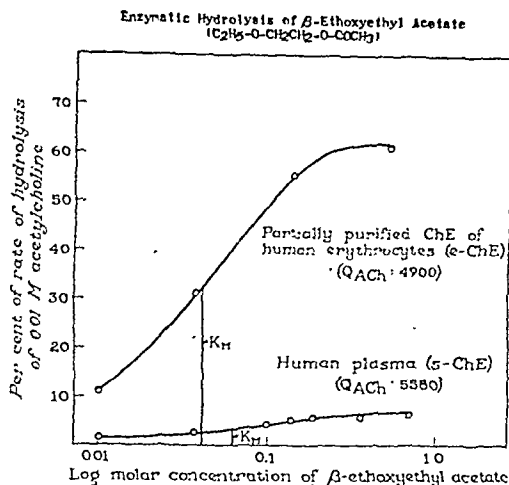


FIG. 1.

Hydrolysis of ethoxyethyl acetate in the presence of human serum and purified erythrocyte cholinesterase; 0.1 ml serum, 0.1 ml purified erythrocyte ChE, total volume 1.0 ml. Reaction velocity expressed in percentage of the reaction velocity caused by 0.01 M acetylcholine.  $K_M$  represents the substrate concentration which produced half of the maximal reaction velocity.

hydrolysis of this ester ( $Q_{DOCA} = 48$ ). On account of the enormous difference between  $Q_{ACH}$  and  $Q_{DOCA}$  no competitive experiments with a mixture of acetylcholine and DOCA were attempted.

**C. Ketopropanol Acetate (KPA).** This substance, which, like DOCA, is a keto-alcohol ester, was attacked by the ester of the venom of *Bungarus fasciatus* ( $Q_{ACH} = 25,000$ ).<sup>5</sup> At 0.2 M concentration the reaction velocity was  $Q_{KPA} = 705$ . Eserine (0.2 mM) and caffeine (0.1 M) depressed the rate 100 and 64 per cent respectively.

**D. Phenyl Acetates.** Phenyl acetate (PA) and various derivatives are hydrolyzed rapidly by preparations of e-ChE and s-ChE (Table II). Like another noncholine ester, ethyl chloroacetate,<sup>4</sup> the hydrolysis of phenyl acetate is inhibited by eserine only in the presence of e-ChE, but not in the presence of human serum. The very rapid hydrolysis of p-nitrophenyl acetate by various snake venoms, by purified erythrocytes ChE and by human serum, and the inhibition of this reaction by the addition of eserine and acetylcholine can easily be followed by the development of the

yellow color, due to the liberation of free p-nitrophenol.<sup>‡</sup>

**Comment.** Besides acetylcholine the acetates of very different hydroxyl derivatives are hydrolyzed by e-cholinesterase ("true" or "specific" ChE). Acetyl derivatives of unsubstituted aliphatic alcohols and halogenated alcohols have been mentioned previously,<sup>1,3,4</sup> while in the present paper representatives of keto-alcohols, poly-alcohols, steroid alcohols and phenols have been added to the list of the noncholine substrates of e-ChE. On the other hand, much less variation is possible on the part of the acyl group, as has been pointed out by the preliminary results obtained by Bovet Nitti,<sup>8</sup> by Adams and Whittaker<sup>9</sup> and by our laboratory. Of the unsubstituted aliphatic acids acetic acid gives the best results, while acids with more than 3 carbon atoms are not as well hydrolyzed. This behavior separates the e-ChE from the s-ChE ("pseudo"-ChE) and from the "ali"-esterase, because these latter enzymes are active toward esters of acids with more than 3 carbon atoms. At the present time the e-ChE can be considered to be an *acetyl*esterase (e-acetylsterase) rather than a *cholin*-esterase.

The ability of the ChE of human erythrocytes to split acetylsalicylic acid and desoxycorticosterone acetate may play a role in the liberation of the corresponding hydroxyl derivatives after the therapeutic application of these and similar substances.

The fact that under certain physiologic and pathologic conditions the ChE activity of a given tissue changed has previously led to far-reaching hypotheses concerning the presence of acetylcholine. Later, similar assumptions were restricted to the e-ChE. In the light of our present results even this latter conclusion can no longer be held. Unless new evidence is produced, no enzymologic clues are available at present to show that acetyl-

‡ The use of phenyl acetate and derivatives thereof for the colorimetric determination of ChE will be discussed elsewhere.

<sup>5</sup> Bovet Nitti, F., *Experientia*, 1947, 3, 283.

<sup>9</sup> Adams, D. H., and Whittaker, V. P., *Biochem. J.*, 1948, 43, xiv.

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Analysis of Chyle for Radioactive Sodium.

Chyle collected from thoracic duct fistulas after injection of isotope into the gastrocnemius muscle.

Dog	Wt, kg	Age of fistula	Chyle collected, cc	Time of collection, hr	Sodium injected, microcuries	% return
1*	11.3	1 day	30	1.5	10	<1
2*	20.0	Immediate postoperative	15	2.5	5†	<1
3	20.0	8 days	30	3	10	<1
4	8.1	1 day	12	3	10	<1
5	10.4	1 day	42	3	15	1.1
6	11.3	1 day post thoracic duct cannulization	16	3.25	13	0.75
7	10.0	1 day	12	1.5	19	<1

\* Anesthetized.

† Injection in left hind and forelegs.

radioactive sodium in the blood draining from the injection site.

**Role of Lymphatics.** The route of egress of any material injected into muscle must be either lymphatic or vascular. Therefore, this portion of the experiment was undertaken to determine if appreciable quantities of the radioactive material could be found in lymph collected from the injected extremity. Although thoracic duct lymph contains lymph from both lower extremities, abdominal viscera, and the left upper extremity, the additional volume and added metabolites found in thoracic duct lymph would not invalidate the accuracy of the counting methods as the presence of the radioactive material could be detected regardless of its dilution. Accordingly, 7 dogs were subjected to operation: thoracic duct fistulas were made in 6, using a modified Biedl technic;<sup>2,3</sup> one additional animal was subjected to thoracic duct cannulation.

**Procedure.** A longitudinal incision was made along the course of the external jugular vein with ligation and division of all tributaries. The thoracic duct was identified as it entered the external jugular vein approximately at its junction with the left subclavian vein. Identification of the duct was facilitated by feeding the dog one pint of milk and cream 30 minutes prior to operation.

The subclavian and innominate veins were

tied and divided below the point of entrance of the thoracic duct, leaving a segment of the external jugular to be used as a conduit to the outside. The vein segment was sutured to the skin, care being taken not to angulate it. A soft rubber catheter was introduced into the fistula and sutured in place; usually the catheter fell away on the first postoperative day.

As it was desirable to collect the chyle from the unanesthetized, ambulatory animal, the following method was devised for this purpose: At the conclusion of the operative procedure, a finger cot, with the end cut off, was sutured around the mouth of the fistula. On the following day, with the animal up and about, another cot was attached by means of Michel clips to the rim of the previously sutured finger cot. This provided a sac into which chyle could drain and which could be changed at periodic intervals.

In one additional dog a polythene cannula was placed in the duct. To protect the cannula, a plaster jacket was placed about the animal's upper thorax, and windows were cut for the cannula and front legs.

Following the animal's recovery from anesthesia, intravenous pentobarbital sodium (Nembutal, Abbott), a 0.1 to 0.2 cc solution of sodium chloride containing 5 to 20 microcuries of radioactive sodium 24 was injected into one of the gastrocnemius muscles. The animal was then encouraged to move about the laboratory, and the thoracic duct lymph was collected for a variable period of time, ranging from 1½ to 3¼ hours. The relative

<sup>2</sup> Biedl and DeCastello, V., *Pflugers Arch.*, 1901, **80**, 259, cited by Lee, F. C., *Bull. Johns Hopkins Hosp.*, 1922, **33**, 21.

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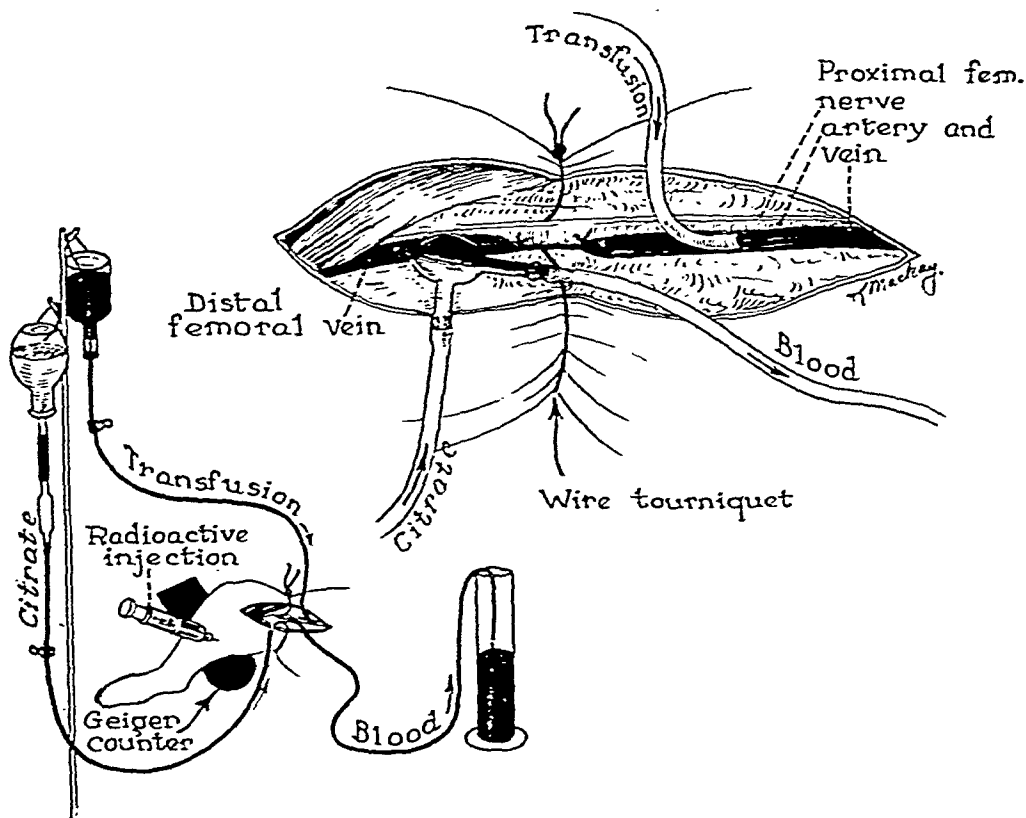


FIG. 1.

a. Geiger-Mueller counter in position to record disappearance of radioactive sodium from site of injection. The femoral nerve, artery and vein are lying above the tightened wire which has compressed and occluded the venous collateral vessels.

b. Relationship of transfusion and collecting cannulas in the femoral vein.

amount of radioactive sodium present in the collected lymph was determined.

As a control, an amount of radioactive sodium equal to that injected was added to a volume of water equal to the volume of chyle collected, and the relative amount of radioactive sodium determined. Correction was made for background radiation, and the relative amounts of sodium in the collected and in the control specimens were compared and expressed in percentage form.

The results of these experiments are recorded in Table I. The 2 anesthetized dogs are included for the purpose of comparing volume of flow in the anesthetized and unanesthetized animals.

*Role of the Capillary and Venous Systems.* The radioactive sodium content of blood re-

turning from the gastrocnemius muscle was studied in 9 dogs. In 2, the procedure was repeated in the opposite leg after an interval of at least 3 days. Therefore, a total of 11 studies was made.

*Procedure.* With the animal under intravenous pentobarbital sodium anesthesia, a longitudinal incision was made over the femoral nerve, artery and vein. These structures were isolated, and a malleable copper wire placed beneath them and brought around the thigh so as to completely encircle it. On tightening the wire, all vessels of the leg, with the exception of the femoral, were compressed; therefore, any sodium being mobilized at the capillary loop and returning to the general circulation had to pass cephalad via the femoral vein.

A Geiger-Mueller counter connected to a

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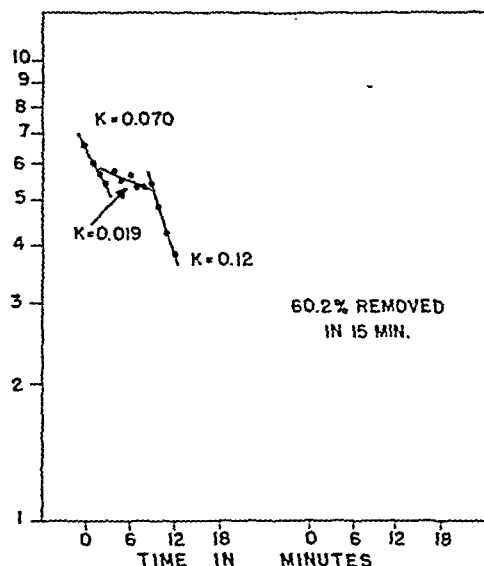
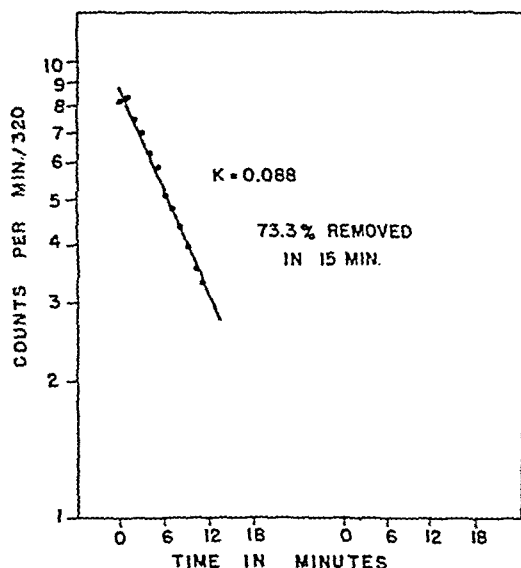


Fig. 2.

Graphs showing rates of mobilization of radioactive sodium from the gastrocnemius muscle of dogs; a, uniform rate; b, fluctuating rate. The line representing the rate of removal is obtained by plotting the counting rate for each minute against time.

site of injection was recorded continuously.

The radioactive content of the collected blood was determined by the same method used for the thoracic duct lymph. The relative amount of radioactive sodium in the collected blood was compared with a control sample also prepared by the same method used for lymph control. The amount of radioactive sodium collected was expressed as a percentage of the amount injected, or, more simply, percentage return in the blood.

The rate of mobilization of the radioactive material from the muscle was determined from the collected blood by the equation:<sup>4</sup>

$$K_1 = \frac{2 - \log(100 - P)}{.4343 T}$$

$K_1$  = Tissue clearance constant as determined from the collected blood.

$T$  = Time elapsed or duration of phlebotomy.

$P$  = Per cent of sodium recovered in the collected blood.

The rate of mobilization was also determined directly at the site of injection by plotting against time on semilogarithmic paper the number of counts for each minute throughout the phlebotomy, as detected by the Geiger-Mueller counter at the injection site. Mathematically, this rate may be expressed by the equation:

$$K_2 = \frac{\log C_2 - \log C_1}{.4343 (T_2 - T_1)}$$

$K_2$  = Tissue clearance constant as determined by measurement directly at the site of injection.

$C_1$  = Number of counts at beginning of phlebotomy or Time  $T_1$ .

$C_2$  = Number of counts at end of phlebotomy or time  $T_2$ .

The results of 11 procedures are shown in Table II. Comparison can be made of the rates of removal ( $K$  values), percentage removed (as measured at the muscle) and percentage returned (as measured from the blood) along with their percentile deviation.

In some instances there was variation in the rate of sodium removal from the site of injection, necessitating multiple determinations of  $K$  value and percentage removed at this site (muscle). Fig. 2 illustrates both a constant and sporadic or irregular rate of mobilization.

**Results.** The lymphatic system of the dog's leg did not play a significant role in the mobilization and removal of the intramuscularly injected radioactive sodium. In no instance was the quantity of radioactive sodium recovered in the thoracic duct lymph greater than 1.1% of the amount injected into the gastrocnemius muscle.



recording mechanism<sup>§</sup> was placed under the leg, just beneath the gastrocnemius muscle. A Jackson cannula was introduced into the distal segment of the femoral vein, and sodium citrate, 2.5%, was allowed to flow in one arm of the cannula and mix with the blood as it left the vein. Attached to the third arm of the cannula was a rubber tube which ran to a collecting flask outside the operative field. To obviate shock, a condition incompatible with normal sodium mobilization, blood from healthy mongrel dogs was given to maintain blood volume. The transfusion technic was to introduce a glass cannula into the proximal end of the femoral vein and replace the blood by way of this cannula. The transfusion was regulated so that blood was replaced at the same rate it was withdrawn. At the moment the collecting cannula was introduced, a saline solution containing 7 to 17 microcuries of radioactive sodium was injected into the gastrocnemius muscle directly over the Geiger-Mueller counter. Fig. 1 illustrates the equipment and methods.

The blood was collected for periods ranging from 10 to 20 minutes. During this time the amount of radioactive sodium present at the

<sup>§</sup> Wire recorder.<sup>4</sup> To simplify the procedure at the time of the experiment and to minimize the factor of human error inherent in recording the counting rate by the usual method, an electro-mechanical system was used in which the impulses from the Geiger-Mueller counter were recorded as they occurred, and analyzed at a later time under more favorable conditions (i.e., in the isotope laboratory rather than in the animal operating room).

A portable wire recorder was used, into which the output of the Geiger-Mueller counter was fed after being passed through an appropriate amplifier. The result was a complete record of the counts as they occurred at the site of injection for the entire period of the phlebotomy.

The wire recording was then fed through an amplifier and conventional scaler, and thence to a translator which drove a mechanical chart recorder. The chart produced was a complete record of the impulses in their order and time of occurrence, from which the counting rate for each minute throughout the run could be computed and plotted.

<sup>4</sup> Kety, S. S., *Am. J. Med. Sciences*, 1948, **215**, 352.

TABLE II.

Isotope injected into the gastrocnemius muscle and wire tourniquet tightened.

Dog	Wt, kg	Estimated* blood volume, cc	Blood collected, cc	Duration of experiment, min.	Sodium injected, microcuries	% removed (muscle)	% collected (blood)	"K" value (muscle)	"K" value (blood)	% deviation
8	15.9	1462	1092	15	11.5	54	49	0.051	0.045	9
9	15.9	1462	750	15	16.5	47	43	0.042	0.038	9
10	8.1	745	250	15	11.4	42	17	0.036	0.012	-60
11	7.7	708	640	20	7.6	52	51	0.037	0.035	2
12	14.0	1288	750	13	16.5	45	46	0.046	0.047	+3
13	12.7	1168	490	18	10.5	80	78	0.089	0.083	-1
14	14.5	1334								
Right leg			615	15	9.4	47	46	0.042	0.041	-2
Left leg			385	15	12.6	60	67	0.061	0.073	+12
15	7.2	662								
Right leg			425	16	8.0	60	65	0.057	0.065	+8
Left leg			400	15	12.9	62	61	0.064	0.063	-2
16	10.0	920	260	15	12.8	50	48	0.046	0.043	-4

\* Blood volume was estimated on the basis of 92 cc/kg of body weight.

# 17246. Is the Salivary *Lactobacillus* Count a Valid Index of Activity of Dental Caries?\*

JULIAN D. BOYD, VIRGIL D. CHEYNE, AND KENNETH E. WESSELS.  
(Introduced by P. C. Jeans.)

*From the Departments of Pediatrics and of Pedodontics, Colleges of Medicine and of Dentistry, State University of Iowa, Iowa City.*

*Lactobacillus* counts are used widely as an index to the activity of tooth decay. Much of the current philosophy relating to the cause and control of dental caries is based on the premise that the *Lactobacillus* count of the saliva provides a dependable prediction medium as to whether the individual in question is likely to develop tooth decay within the reasonably near future. Yet few studies have been reported wherein *Lactobacillus* counts have been correlated individually with the previous and subsequent progress of tooth decay over long periods of time for large numbers of subjects. Studies of that type are needed to show the validity of the *Lactobacillus* count as a diagnostic or prognostic agent. The study reported herewith was designed to supply evidence as to the significance of the *Lactobacillus* count to the individual subject.

As a part of a larger and more general

survey, the *Lactobacillus* counts from the saliva of 64 teen-aged girls have been compared with the progression of tooth decay observed for not less than 30 months for each individual subject. The data include 407 separate *Lactobacillus* counts. The dental examinations were made recurrently from July, 1946 to January, 1949; the *Lactobacillus* counts were made on successive occasions from March, 1948 until January, 1949. The State Hygienic Laboratories made the bacterial counts, as a part of the service they provide to the dentists of the state for clinical diagnosis and prognosis.

Generally speaking, there was a slight trend toward parallelism of the *Lactobacillus* counts and rates of progression of caries when massed data were used. However, when the group as a whole was subdivided according to the rate of caries progression, there was little difference between the range of *Lactobacillus*

TABLE I.  
*Lactobacillus* Counts from 64 Subjects Observed Serially for 2½ Years.

	(a) those with no advance of caries	(b) those with greatest advance	(c) total group
No. of subjects	11	15	64
No. of counts	68	105	407
Mean number of <i>Lactobacilli</i> per cc	92,500	112,000	104,500
S.D.	70,000	106,300	88,500
S.E. mean	8,500	10,400	4,040
Decile rating:			
10th	30,000	35,000	30,000
20th	37,000	48,000	43,000
30th	46,000	60,000	50,000
40th	60,000	65,000	60,000
50th	70,000	80,000	72,000
60th	92,000	96,000	90,000
70th	100,000	125,000	120,000
80th	149,000	150,000	144,000
90th	180,000	200,000	180,000
100th	360,000	720,000	720,000
Avg. No. of new DMF tooth surfaces per annum	None	more than 3.5	2.12

\* This study was made possible through a grant from the Sugar Research Foundation.

The data obtained on the group of dogs in which the collateral venous system of the thigh was occluded by a wire tourniquet (Table II) shows good correlation between the percentage known to have been mobilized and the percentage actually recovered in the blood draining the limb.¶

Failure to recover 60% of the isotope in the case of dog No. 10 may be attributed to one or both of the following factors: It is possible that the wire was not tightened sufficiently to occlude completely the collateral channels; it is possible that a thrombus might have partially occluded the lumen of the canula. A thrombus would prevent a free flow of blood, resulting in stasis, dilatation of the femoral vein and tributaries, and increased venous pressure. The volume of blood collected in this dog was low, a finding which tended to substantiate the latter hypothesis.

The remaining 10 analyses in this series showed percentage deviations within 12%. Control studies showed that a 12% error was possible with this technic.¶ Therefore, it was concluded that radioactive sodium could be successfully recovered from the blood draining the injection site and that the mobilization of this material was solely a function of

the capillary loop. In view of the fact that the isotope was not found in the thoracic duct lymph, and that it was recovered quantitatively in the blood, the factors of lymphatic removal and diffusion beyond the range of the Geiger-Mueller counter appeared to be insignificant. The assumption that removal of sodium 24 was a function of the hemovascular system, and consequently, was dependent upon the state of the circulation at the site of injection, was apparently valid.

*Summary.* A study of sodium mobilization from the gastrocnemius muscle of dogs by the use of radioactive sodium 24 is reported.

The sodium content of thoracic duct lymph following injection of sodium 24 into the gastrocnemius muscle was measured in 7 dogs to determine the role of the lymphatic system in the mobilization of the sodium.

The capillary mobilization and venous transport of sodium 24 from the gastrocnemius muscle was studied in 11 extremities. The radioactive sodium content of the blood draining from the gastrocnemius muscle was determined by two methods: (1) directly, by actual measurement of the sodium 24 content of collected blood, and (2) indirectly, by means of a Geiger-Mueller counter placed beneath the site of injection of the radioactive sodium into the muscle.

*Conclusions.* The lymphatic system of the dog's hind extremity does not play a significant role in the mobilization of intramuscularly injected sodium, nor does the radioactive material diffuse along intramuscular planes beyond the range of the Geiger-Mueller counter.

Intramuscularly injected radioactive sodium can be quantitatively recovered from femoral vein blood if the collateral venous system of the upper thigh is occluded.

The rate of removal of sodium 24 measured external to the site of injection is the same as that determined from the blood actually draining from the muscle.

The authors wish to express their appreciation for the technical assistance given by Mr. Richmond Waits.

¶ To determine the percentage of error inherent in the counting technic used in this experiment, equal quantities of sodium 24 in the form of sodium chloride were placed in equal volumes of water and blood, and counting rates determined. It was found that the percentage of error in counting rates between the two fluids varied from 2 to 12%. Accordingly, the results have been interpreted in the light of a possible 12% deviation.

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group for the diagnosis or the prognosis of caries activity for the individual subject.

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## 17247. Nucleoproteins and the Cytological Chemistry of *Paramecium* Nuclei.\*

MONTROSE J. MOSES. (Introduced by A. J. Goldforb.)

From the Department of Zoology, Columbia University, and the Department of Biology, Brookhaven National Laboratory.

There are many exceptions to the classical definition of a cell as "a mass of protoplasm containing a nucleus."<sup>1</sup> Numerous examples are known of binucleate and multinucleate protoplasmic masses. In most cases the nuclei are all alike, but in binucleate ciliated protozoa such as *Paramecium*, the two nuclei are markedly different in size, and are therefore known as the macronucleus and the micronucleus. There are also considerable differences in behavior of these two nuclei. It is the general view that the macronucleus is a "somatic nucleus" serving the indispensable physiological needs of the organism in vegetative stages when no detectable effect of the micronucleus can be observed.<sup>2</sup> The micronucleus appears to be of chief importance as a potentially germinal structure, functioning in the nuclear interchange and reorganization which take place at conjugation or autogamy.<sup>2</sup> The present paper briefly reports the results of a study of the nucleoprotein composition of macro- and micronuclei in vegetative individuals of *Paramecium*; experiments were designed to answer the question whether in these stages the two nuclei are as unlike as other experimental evidence indicates them to be.

**Experimental.** The chemical constituents of nuclei are mainly nucleoproteins, and the

quantitative technics of cytological chemistry are particularly suited to analysis and localization of these substances within individual nuclei. The procedures used in this investigation permit the computation of relative concentrations of nucleic acids and proteins from specific chemical reactions which have been shown to be applicable to properly fixed cytological preparations. Analyses were made by microscopic photometry of total and non-histone protein from modifications of the Millon reaction,<sup>3,4</sup> of desoxyribose nucleic acid (DNA) from the Feulgen nuclear reaction,<sup>5</sup> of total nucleic acids and polynucleotides (removable with hot trichloroacetic acid) from the absorption in the ultraviolet (near 260 m $\mu$ ) of their purine and pyrimidine bases<sup>6,3</sup> and of ribose nucleic acid (RNA) by difference in absorption at 260 m $\mu$  after enzymatic digestion with protease-free ribonuclease.<sup>7</sup>

Determinations were made on a culture of *Paramecium caudatum* which was raised on a baked lettuce medium inoculated with *A. aerogenes*<sup>8</sup> and kept under constant feeding

\* Part of the research reported here was carried out at Brookhaven National Laboratory under the auspices of the Atomic Energy Commission.

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<sup>6</sup> Caspersson, T., *Skand. Arch. Physiol.*, 1936, 73, suppl. S, 1.

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<sup>8</sup> Sonneborn, T. M., and Dippell, R., *Physiol. Zool.*, 1946, 19, 1.

## SIGNIFICANCE OF LACTOBACILLUS COUNTS

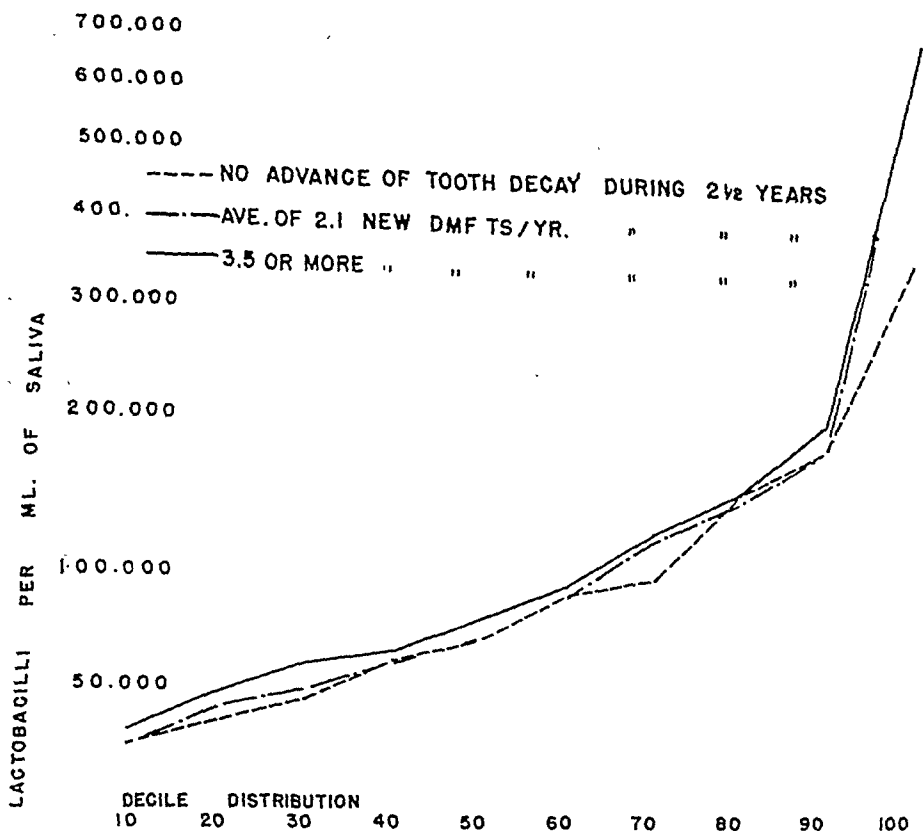


FIG. 1.

Decile range for Lactobacillus counts observed from saliva of teen-aged girls whose rates of progression of tooth decay (number of newly affected tooth surfaces: decayed, missing or filled: per annum) were observed individually for not less than 30 months. Separate curves are shown for 11 girls with zero progression, for the 15 girls with the most rapid progression, and for the total group of 64 subjects. The hundredth per centile represents the highest value observed for each sub-group.

counts observed among those with the least and those with the greatest progression of tooth decay.

Eleven of the girls showed no measurable progression of tooth decay throughout the period of more than 2½ years, when scored according to the number of new decayed, missing or filled (DMF) surfaces which developed during the interim. The range of the Lactobacillus counts observed from these girls' saliva samples has been contrasted with the range for the entire group of 64 subjects, and also with the range observed among the 15 girls whose caries progression rates were the highest. The results are summarized in Table I and Fig. 1.

Emphasis is directed toward the high Lactobacillus counts observed among those children who had had no progression of tooth decay during the 20 months which preceded their first Lactobacillus count, and who continued free from evidence of caries advance at least for the 10 months which followed. Obviously the persistence of high Lactobacillus counts in these children's mouths had not led to the initiation of tooth decay during the period of observation. Moreover, for these 11 girls out of a group of 64 subjects the Lactobacillus count failed to serve as a proper index of caries susceptibility. Furthermore, analysis of the remainder of the group shows such inconstancy of relationship between the magni-

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TABLE I.  
Comparison of Nucleic Acid and Protein Contents of the Macro- and Micronucleus of  
*Paramecium caudatum*.

	Total protein	Desoxyribose nucleic acid	Ribose nucleic acid
A. % macronuclear nucleoprotein determined	85.1	5.8	9.1
B. % micronuclear nucleoprotein determined	87.6	4.2	8.2
C. Ratio concentrations: <u>Macronucleus</u>	0.9	1.2	1.0
<u>Micronucleus</u>			

and environmental conditions. At no time during the life of the culture was autogamy or conjugation observed; all individuals were in a "vegetative" state. The culture was concentrated by mild centrifugation, fixed in Carnoy's 1:3 acetic acid-alcohol, transferred to small agar blocks and imbedded in paraffin. Analyses were carried out on serial sections mounted on slides in the usual manner.

Mean extinction values ( $\log_{10} I_0/I$ ) were computed from transmission measurements of cylindrical areas of a large number (30-50) of random sections of macronuclei, and of 8-12 entire micronuclei. The results were fairly uniform, as shown by the fact that the standard errors in no case exceeded 7% of the mean extinctions. From these mean values concentrations were computed by reference to standards measured on known dilute solutions in a Beckman Spectrophotometer. An approximation to the protein concentration was computed by assuming that the Millon reaction was due to a tyrosine derivative which constituted one-sixteenth (6.25%) of the protein. The total nucleoprotein concentration was computed by adding together the mean concentrations of protein, desoxyribose nucleic acid (DNA), and ribose nucleic acid (RNA). Table I (A and B) shows the percentages of the total concentration represented by each of these components.

**Results.** It is apparent from the data that protein is the major component of the nucleus (Table I), comprising over 85% of the nucleoprotein determined for each nucleus. A comparison of the extinctions of nuclei prepared to show total and non-histone proteins

showed no significant difference and it followed that histone could not have comprised more than a few percent of the nucleoprotein. The protein/DNA ratio was from 15-20 to 1, in the range of high values reported by Pollister and Leuchtenberger<sup>4</sup> for metabolic nuclei of metazoa. The concentration of DNA present in the nuclei was quite comparable with determinations of other workers, being in the order of  $10^{-12}$  mg per cubic micron. Most remarkable, however, are the results of digestion with purified crystalline ribonuclease<sup>9</sup> which show that about 60% of the total nucleotide extinction was lost after treatment and that a large quantity of RNA was consequently present. The RNA released by the enzyme amounted to almost twice the DNA present in the nucleus and comprised about 10% of the nucleoprotein moiety. That RNA might be contained in the macronucleus of *Paramecium* has already been indicated by the staining experiments of Shubnikova<sup>10</sup> and others who, with methyl green and pyronin (unpurified), showed pyronin coloring in the macronucleus removable with ribonuclease (unpurified). RNA is known to be a component of metazoan nuclei both in the nucleolus and in the chromosomes. Mirsky has indicated by his analyses of isolated mammalian chromosomes that the RNA which is a component of the residual chromosome, is high in active metabolizing cells and low in relatively inactive ones.<sup>11</sup>

**Discussion.** It can be seen from the ratios

<sup>9</sup> McDonald, M., *J. Gen. Physiol.*, 1948, **82**, 33.

<sup>10</sup> Shubnikova, E., *C. R. Acad. Sci. URSS*, 1947, **55**, 517.

of concentrations in Table I, row C, that the macro- and micronuclei are very much alike in nucleoprotein composition. It follows, then, that insofar as their chemical composition is indicative of their function, both nuclei are performing similar roles in the vegetative individual. Thus, the organism's dependence on the macronucleus and its apparent independence of the micronucleus during these stages<sup>2</sup> simply becomes a matter of the preponderance of the physiological-genetic material in the large macronucleus. There is a marked resemblance between these protozoan nuclei and highly metabolic nuclei of metazoa, as typified by the mammalian liver nucleus, which is strong evidence for the active participation of both macro- and micronucleus in metabolic functions of the cell. Any chemical

differences which may serve to be correlated with the capacity of the micronucleus to differentiate into a special reproductive structure must be looked for at some other time in the organism's history. Presumably this is during the reproductive processes of conjugation and autogamy, when the behavior and morphology of the two nuclei are most markedly dissimilar.

*Summary.* Quantitative cytological chemical analyses of the nucleoprotein composition of the macro- and micronuclei of a clone of *P. caudatum* show that both nuclei contain protein, RNA and DNA in the ratio of 20:2:1. Twice as much RNA as DNA was found. From their resemblances to the nucleoprotein contents of metabolic metazoan nuclei, it is concluded that in the vegetative stage, both nuclei of *P. caudatum* are actively metabolic.

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<sup>11</sup> Mirsky, A. E., *Cold Spring Harbor Symposium on Quant. Biol.*, 1947. 12, 143.

## 17248. Influence of Level of Adrenal Cortical Steroids on Sensitivity of Mice to X Irradiation.

R. L. STRAUBE, H. M. PATT, E. B. TYREE, AND D. E. SMITH.

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We reported previously that X radiation appeared to result in an increased demand for the adrenal cortical hormone.<sup>1</sup> Subsequent studies indicated that the adrenal changes noted after total-body X irradiation of rats (decreased cholesterol content and increased weight) were mediated by the pituitary.<sup>2</sup> The adrenal response could be prevented, in part, by suitable administration of adrenal cortical extract. However, such treatment failed to alter survival.<sup>3</sup> Since Ellinger<sup>4</sup> reported earlier that the daily injection of desoxycorticosterone increased survival of ir-

radiated mice, it seemed desirable to extend these observations in order to clarify the role of the adrenal in the acute radiation syndrome. In these experiments we have determined the radiosensitivity of intact mice with and without whole adrenal cortical extract or desoxycorticosterone. Data on the sensitivity of adrenalectomized mice which were obtained as part of another study have been included for comparison.

*Materials and Methods.* Male and female CFl mice, weighing 18 to 28 g each, were the experimental animals. Mice of the same sex were used in individual experiments. For the irradiation, the animals were placed in individual cellulose acetate exposure cells and were given total-body exposures in groups of 16. The cells were rotated slowly on an electrically-driven turntable to insure equivalent irradiation of all the animals. Equal numbers of control and experimental mice

<sup>1</sup> Patt, H. M., Swift, M. N., Tyree, E. B., and John, E. S., *Am. J. Physiol.*, 1947. 150, 480.

<sup>2</sup> Patt, H. M., Swift, M. N., Tyree, E. B., and Straube, R. L., *Science*, 1948, 108, 475.

<sup>3</sup> Swift, M. N., Patt, H. M., and Tyree, E. B., *Fed. Proc.*, 1948, 7, 121.

<sup>4</sup> Ellinger, F., *Proc. Soc. Exp. Biol. and Med.*, 1947, 64, 31.



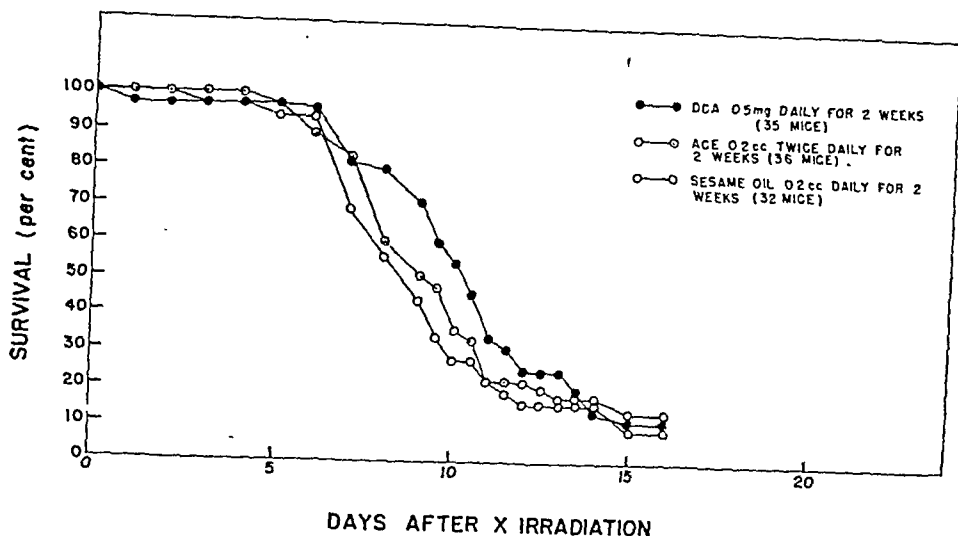


Fig. 1.

Effect of desoxycorticosterone (DCA) and whole adrenal cortical extract (ACE) on radiation mortality in mice (CF1 males, 500 r).

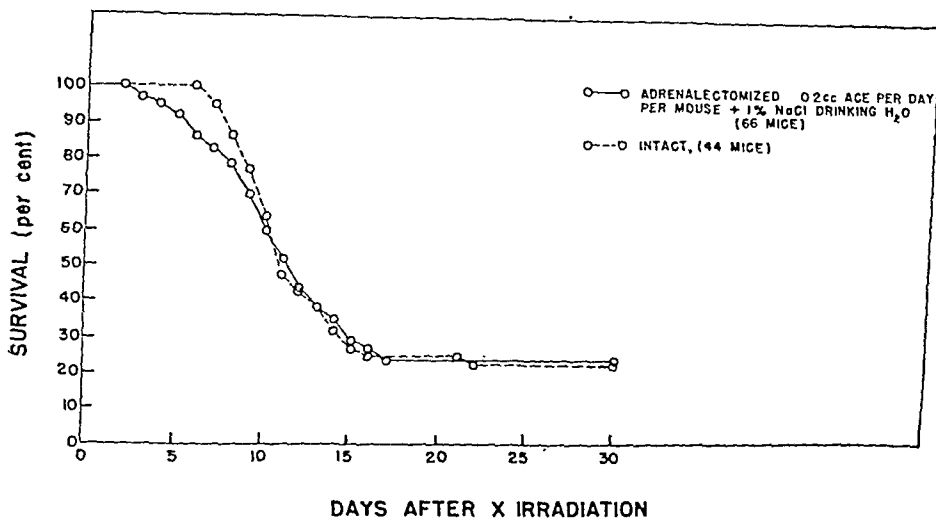


Fig. 2.

Effect of whole adrenal cortical extract (ACE) on radiation mortality in adrenalectomized mice (CF1 males, 500 r).

were used for each exposure. The radiation factors were: 200 kv, 15 ma, 0.5-mm Cu and 3.0-mm Bakelite filters, HVL-1.20-mm Cu, target distance, 10.8 cm, and dose rate, 20 r per minute. Male mice received 500 r and females, 550 r (measured in air).

Single stage bilateral adrenalectomies were performed under nembutal anesthesia 15 to 17 days prior to irradiation. All animals received a postoperative injection of 0.2 cc

whole adrenal cortical extract (Wilson).

Survival of irradiated mice was compared under the following 4 experimental conditions:

1) Mice injected intramuscularly with desoxycorticosterone acetate (Schering, 0.5 mg daily) for 2 weeks after irradiation. The irradiated control group received equivalent amounts of sesame oil.

2) Mice injected subcutaneously with whole

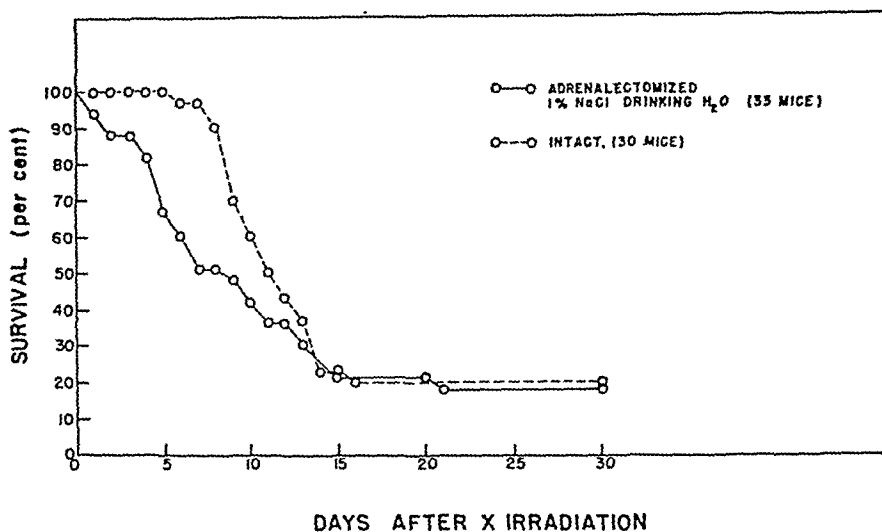


FIG. 3.

Effect of adrenalectomy on radiation mortality in mice (CFL females, 550 r).

adrenal cortical extract, 0.2 cc, twice daily, for 2 weeks following X irradiation. The control group in this instance was identical with that of 1) above.

3) Bilaterally adrenalectomized mice maintained on salted drinking water (1 per cent NaCl) and daily injections of 0.2 cc whole adrenal cortical extract. Intact irradiated mice were used as controls.

4) Bilaterally adrenalectomized mice maintained on salted drinking water alone, plus an intact irradiated control group.

**Results and Discussion.** As may be noted in Fig. 1 and 2, mice that received desoxycorticosterone acetate or whole adrenal cortical extract, as well as those that were adrenalectomized and maintained on whole adrenal cortical extract plus salt exhibited a final radiation mortality identical with that of intact irradiated controls. Although there was a decreased mean survival time ( $p < 0.001$ ), those adrenalectomized animals maintained on salted drinking water alone (Fig. 3) also show a final mortality similar to that of the normal irradiated controls and the 3 experimental groups noted above.

The schedule of treatment used in administering desoxycorticosterone is that reported by Ellinger to yield maximal protection in terms of survival.<sup>4</sup> We are unable to account for the failure of desoxycorticosterone to modify survival in our studies. This disparity in results may reside in the different strain of mice used. It is of interest to note that both larger and smaller doses of desoxycorticosterone were ineffectual according to Ellinger.

From these data we may conclude that the survival of X-irradiated mice appears to be independent, at least within certain limits, of the amount of adrenal cortical steroids present. The adrenals are apparently involved only secondarily as part of the organism's buffer response to the stress of irradiation. The rationale of employing adrenal corticoids to alter radiation mortality is therefore open to question.

**Summary.** The experiments cited indicate that the radiosensitivity of intact or adrenalectomized mice, with or without exogenous adrenal cortical steroids, is similar.

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## 17249. Réactions of Normal Ovaries to Injections of Stilbestrol.\*

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It is a common belief that injected hormones do not stimulate the organs which normally produce them. While injected estrogens may have no direct stimulatory effect on ovarian follicles of normal adult animals, they do produce certain ovarian changes either by direct action on the ovaries, or indirectly via the anterior pituitary gland.<sup>1,2</sup> The consensus is that, if the injected doses are of sufficient potency, there will be (1) luteinization,<sup>3-5</sup> and (2) inhibition or depression of follicular development,<sup>2,6,7</sup> and that these effects are secondary to pituitary changes produced by increased estrogen. It is probable that FSH normally stimulates medium-sized follicles to develop fully to the ovulating stage. If the estrogen level goes over a certain point, the production of FSH is inhibited or depressed, thus resulting in failure of the development of ovulating follicles. At the same time, LH is liberated, either as a result of inhibition of FSH or due to the direct stimulatory effect of increased estrogen, and LH stimulates luteal formation in the ovary. There is the possibility that luteinization is an effect of increased estrogens acting directly on the ovaries, but assays of pituitary

glands after estrogenic treatment show an increased potency of luteinizing function.<sup>8</sup>

*Materials and methods.* Follicular development and luteinization in the ovaries of normal adult rats were studied after intraperitoneal injections of large doses of the synthetic estrogen, diethylstilbestrol dipropionate ("Estrobene DP"). Inbred Sprague-Dawley rats were used, and the work was done on 100-day-old virgin females. The ovaries of 11 control rats were removed at the 5 stages of the estrous cycle and were serially sectioned. In the experimental group, consisting of 15 rats, 5 mg of stilbestrol were injected intraperitoneally once per day, ranging from 1 to 12 injections. All of the injections were begun at diestrus. The ovaries were removed 24 hours after the last scheduled injection, were fixed and serially sectioned. From the serial sections of the control and experimental groups the following determinations were made: (1) the total volumes of the ovaries were determined by planimeter measurements of serial projection drawings; (2) the total volumes of the luteal tissue were measured by the same methods, and the percentage luteinization determined in each case; (3) the follicles were counted and divided into 4 categories: normal and atretic small follicles, and normal and atretic follicles in which an antrum had definitely formed.

*Results.* In the results, which are summarized in Table I, the animals are divided into 4 groups, and the average figures are given for each group. Group A consists of the 11 control animals in the various stages of the normal 4-day estrous cycle. Groups B, C, and D consist of 15 animals which received daily injections of 5 mg of diethylstilbestrol over a period ranging from 1 to 12 days. In Group B, the animals received 5 to 20 mg in the 4-day period ranging from 1 to 4 days; in Group C, 25 to 40 mg in the

\* This work was aided by the fund contributed by the M. D. Anderson Foundation. The author wishes to express his appreciation for the excellent technical assistance of Miss Sarah Lea O'Neill.

<sup>1</sup> Kunde, M. M., D'Amour, F. E., Gustavson, R. G., and Carlson, A. J., *Proc. Soc. Exp. Biol. and Med.*, 1930, **28**, 122.

<sup>2</sup> Moore, C. R., and Price, D., *Am. J. Anat.*, 1932, **50**, 13.

<sup>3</sup> Fevold, H. L., Hisaw, F. L., and Greep, R., *Am. J. Physiol.*, 1935, **114**, 508.

<sup>4</sup> Desclin, L., *Compt. rend. Soc. de biol.*, 1935, **120**, 526.

<sup>5</sup> Meyer, R. K., and Hertz, R., *Am. J. Physiol.*, 1937, **120**, 232.

<sup>6</sup> Allen, E., and Diddle, A. W., *Am. J. Obst. and Gynec.*, 1935, **29**, 83.

<sup>7</sup> Bullough, W. S., *J. Endocrinology*, 1943, **3**, 235.

<sup>8</sup> Lane, C. E., *Am. J. Physiol.*, 1934, **110**, 681.

TABLE I.  
Summary of Effects of Stilbestrol on the Ovary.

Determinations	Group A, control, avg	Group B, 1-4 day stilbestrol, avg	Group C, 5-8 day stilbestrol, avg	Group D, 9-12 day stilbestrol, avg
a. Volume of ovaries in 1000 planimeter units	46.6	39.8	44.9	56.9
b. Volume of corpora lutea in 1000 planimeter units	20.7	16.1	21.2	30.4
c. % luteinization	44.2	40.1	45.8	53.0
d. Total No. of follicles	435	292	235	281
e. Total No. of normal follicles	334	229	187	190
f. Total No. of atretic follicles	101	62	48	91
g. No. of normal small follicles	259	159	97	75
h. No. of atretic small follicles	61	36	18	24
i. No. of normal antrum-containing follicles	75	70	80	115
j. No. of atretic antrum-containing follicles	40	26	30	67

4-day period ranging from 5 to 8 days; and in Group D, 45 to 60 mg in the 4-day period ranging from 9 to 12 days.

With progressive injections of stilbestrol there is an initial decrease in ovarian size during the first 6 days, followed by a marked increase over the normal size (Table I, a). Similarly with luteal tissue (Table I, b), there is an initial decrease followed by a marked increase in luteinization, which is directly proportional to the increase in ovarian size. The degree of luteinization is shown in Table I, c. During the stilbestrol injections there is an initial 4-day lag followed by a marked increase in the degree of luteinization. The initial lag may be due to a natural hypophyseal-gonadal lag, which has been indicated by the 2-4-day delay in the effect of hypophysectomy on ovaries.<sup>9</sup>

Following treatment with stilbestrol there is a significant decrease in the total number of follicles from the second day on (Table I, d). This consists of a decrease in the number of both atretic and normal follicles (Table I, e,f), with some recovery in the number of atretic follicles towards the end of the experimental period. If there is a

complete physiological block of FSH after injections of massive doses of stilbestrol, one would expect the follicular picture to resemble that after hypophysectomy, except for any direct effects stilbestrol may have on the ovaries. Smith<sup>9</sup> has described the follicular picture after hypophysectomy in the rat as follows: (1) all medium-sized and large follicles (*i.e.*, all antrum-containing follicles) become atretic within 4 days or less, and formation of normal follicles in this group is terminated; (2) small follicles continue to develop, but become atretic before antrum formation. In an attempt to analyse the effects of large doses of stilbestrol, the follicles in these two categories were counted and the number of normal and atretic follicles determined in each.

It is evident that there is a continuous and rapid decline in number of normal small follicles with progressive stilbestrol injections (Table I, g). Similarly, as shown in Table I, h, there is a decrease in the number of atretic small follicles. This decrease in the number of small follicles indicates that in our series

<sup>9</sup> Smith, Philip E., *Am. J. Anat.*, 1930, 45, 205.

there is an inhibition or depression of the formation of small follicles which is a direct effect of stilbestrol on the ovary. Table I, i shows that, after a slight initial depression during the first 4 days of injection, there is an increase in the number of normal antrum-containing follicles. This increase indicates that follicular development still proceeds from the small to the antrum-containing stage. This again differs from the results of hypophysectomy where there is a complete absence of normal antrum-containing follicles within 4 days after operation, and indicates that FSH is not completely blocked by massive doses of stilbestrol. Table I, j shows that there is an initial decrease in the number of

atretic antrum-containing follicles, followed by a marked increase towards the end of the experimental period. This indicates that the follicle-stimulating function is depressed to some extent. The delay in the appearance of increased atresia argues against a direct effect.

*Summary.* These results support previous evidence that the ovarian structures which normally secrete estrogen, *i.e.*, antrum-containing follicles and corpora lutea, are affected when the estrogen level is raised, presumably via the anterior hypophysis. However, the suppression of formation of new small follicles is evident as a direct effect of stilbestrol on the ovary.

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### 17250. Male Mice Tolerate Dosages of Pteroylglutamic Acid\* Lethal to Females.

ALFRED TAYLOR AND NELL CARMICHAEL.

*From the Biochemical Institute, University of Texas, and the Clayton Foundation for Research, Austin, Texas.*

Female mice are much more susceptible than males to high dosages of pteroylglutamic acid (folic acid). Male mice easily tolerate amounts of this material lethal to every female injected. Females receiving sublethal levels of the compound lose more weight and are slower to recover than comparably treated males.

Harned<sup>1</sup> and associates have investigated the pharmacology of folic acid. They reported this compound to be comparatively innocuous at levels much above the therapeutic range.

When 100 to 400 mg per kg were administered intravenously, acute toxicity was observed in mice, rats, rabbits, and guinea pigs. Death was apparently due to obstruction of renal tubules as a result of the precipitation of folic acid. The mouse and the rat were more resistant than the guinea pig and the rabbit.

There is no mention in the paper of a sex difference in the response of the animals to the material injected.

*Experimental.* More than 400 mice of the dba strain were used in these experiments.

Folic acid was administered both in the form of a saline suspension (0.85%) and dissolved in a solution of sodium bicarbonate (3%). The saline suspension was most frequently used. The various amounts given were diluted so that each animal received 0.1 to 0.2 cc per injection. The dosages given were based on a 25 g mouse unless otherwise stated.

Healthy mature dba male and female mice were subjected to single subdermal injections of 1 to 40 mg (saline suspension) of folic acid to test the effect of massive doses on mortality. The results are summarized in Table I.

Autopsy disclosed the usual precipitation of folic acid in the renal tubules as indicated by the yellowish color of the kidneys. The spleen was reduced in size. Weight averages

\* Pteroylglutamic acid for this research was furnished by Lederle Laboratories, Inc.

<sup>1</sup> Harned, B. K., Cunningham, R. W., Smith, H. D., and Clark, M. C., *Ann. N. Y. Acad. Sci.*, 1946, 48, 289.

TABLE I.  
Effect of a Single Injection of Folic Acid on Mortality of Male and Female dba Mice.

Dosage (mg per 25 g mouse)	Male		Female	
	No. injected	No. dead	No. injected	No. dead
1	3	0	3	0
3	3	0	3	0
5	10	1	15	3
10	8	2	11	9
15	7	0	7	7
20	8	0	8	8
40	4	0	4	4

of spleens from female mice receiving 10 mg of folic acid and spleens from control females showed an average reduction of 40% in the spleen weights of the experimental mice. The visceral effects in the male were similar to those observed in the female.

Male and female mice of the same initial body weight were given sublethal injections of folic acid and the effect on the body weight recorded over a period of time.

One group consisted of 20 male and 20 female mice not yet fully mature so that the initial body weights could be exactly matched. Each mouse received a single injection of 5 mg folic acid. The females showed a 10% loss in body weight and had not completely recovered 12 days after the administration of the compound, while the males showed only slight loss of weight followed by rapid recovery and gain in weight.

In other tests mature male and female mice manifested the same sex difference in their response to sublethal injections of folic acid.

In one experiment 6-week-old male and female mice were given single injections of 5 mg folic acid. The males continued to gain

weight but more slowly than normal. They gained an average of 3 g in the week following the treatment. The females, one week after the injection weighed an average of 1 g less than their initial weight indicating they were seriously affected.

*Discussion.* Why the female should be so much more susceptible than the male to high dosages of folic acid has not been determined.

So far as could be discovered no other compound has ever been reported which manifests such a sex difference in its pharmacology.

*Summary and conclusion.* Male mice are much more resistant than female mice to injections of high dosages of folic acid.

Male mice were only slightly affected by single subdermal injections of 5 mg of folic acid. Female mice receiving the same dosage averaged a 10% loss in body weight followed by a slow recovery.

There were no deaths in a group of male mice receiving 15 to 40 mg in a single injection of folic acid. The same dosages administered to female mice were rapidly lethal in every instance.

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<sup>1</sup> Harned, B. K., Cunningham, R. W., Smith, H. D., and Clark, M. C., *Ann. N. Y. Acad. Sci.*, 1946, 48, 289.

TABLE I.

Precipitin Tests Performed upon Rat Urine after Parenteral Injection of Bovine Albumin.

Rat No.	Total protein excretion, mg/24 hr	Urine dilution	Precipitin tests	
			Bov. alb. antiserum 0 to 4 +	Rat serum antiserum 0 to 4 +
62	842	1:24	4	4
35	1440	1:40	1	2
61	859	1:24	2	2
80	320	1:8	2	2
53	750	1:16	2	2
00	106	1:20	3	1
02	202	1:50	2	2
04	404	1:100	3	2
08	270	1:50	4	2
15	1197	1:200	4	1
17	414	1:100	3	2
21	274	1:50	4	1
25	151	1:20	3	2
30	548	1:100	4	1
35	358	1:50	3	2
50	529	1:100	3	2
65	468	1:100	3	2

TABLE II.

Precipitin Tests Performed upon Rat Urine after Parenteral Injection of Egg Albumin.

Rat No.	Total protein excretion, mg/24 hr	Urine dilution	Precipitin tests		Urine dilution	Precip. test rat serum antiserum 0 to 4 +
			Egg alb. antiserum 0 to 4 +	Rat serum antiserum 0 to 4 +		
38a	151	1:20	3	0		
26	158	1:20	2	0		
29	168	1:20	3	0		
31	145	1:20	3	0		
32	139	1:10	3	0		
59	188	1:6	4	0		
25	258	1:250	3	0	1:10	0
38b	88	1:100	2	0	1:10	0
67	234	1:250	4	0	1:10	0
64	195	1:200	3	0	1:10	0
51	80	1:100	4	0	1:10	0
72	192	1:200	3	0	1:10	1
20	208	1:200	4	0	1:10	1
92	139	1:150	3	0	1:10	1
24	90	1:100	3	0	1:10	1
77	18	1:20	3	0	1:10	0
52	184	1:200	4	0	1:10	0
22	225	1:200	3	0	1:10	1

As the light absorption coefficient is specific for each protein, it is necessary to determine the coefficient for each protein or mixture in constant ratio that is used. It was found that the degree of absorption for bovine albumin and egg albumin bore a linear relation to concentration, in accordance with Beer's Law. Highly variable results were obtained with the precipitates formed with rat serum antigen. This was attributed to in-

homogeneity of the antigen, and this antiserum was not used for quantitative determinations.

Urine sample were tested qualitatively by precipitation with the appropriate antiserum in the zone of marked antibody excess. For quantitative determinations, precipitation was performed as described, the precipitate was dissolved in 4 cc of 4% sodium hydroxide, after washing and draining, and the light



# 17251. Mechanism of Proteinuria. II. Identity of Urinary Proteins in the Rat Following Parenteral Protein Injection.

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In considering the proteinuria produced by Addis and associates<sup>1</sup> in the rat by intraperitoneal injections of protein, it seemed logical to conjecture that the urinary protein might be identical with the injected protein. Thus, after injections of bovine albumin, it was supposed that the urinary protein, appearing after large doses and some lapse of time, was largely bovine albumin. On the other hand, after injections of egg white and Bence-Jones protein, it was conjectured that the small molecules might more easily and quickly permeate the glomerular membrane, so that protein appeared in the urine rapidly and after smaller dosage.

Doubt was cast upon this desirably simple hypothesis by work recently reported<sup>2</sup> which showed, by the use of hemoglobin as an indicator, that administration of bovine albumin, under the conditions used by Addis, not only saturated the tubular capacity for reabsorbing hemoglobin, but also altered the glomerular permeability to hemoglobin. In contrast,<sup>3</sup> administration of egg white affected hemoglobin excretion in a very different manner. In this study it was possible to demonstrate a similar functional difference between the effects of bovine albumin and egg albumin upon rat serum protein excretion.

\* The author gratefully acknowledges the technical assistance of Helen J. Ureen and Natalie Stein. This work was supported by a grant from the Division of Research Grants and Fellowships, National Institutes of Health, United States Public Health Service, and a grant from the Columbia Foundation, San Francisco, Calif. Bovine albumin (Fraction V) was furnished through the courtesy of Dr. J. D. Porsche, Armour Laboratories, Chicago, Ill.

<sup>1</sup> Addis, T., Final Report, Contract 338, Office of Scientific Research and Development, Committee on Medical Research, 1946.

<sup>2</sup> Lippman, R. W., *Am. J. Physiol.*, 1948, **154**, 532.

<sup>3</sup> Lippman, R. W., data to be published.

*Methods.* Rabbit antisera to bovine albumin, egg albumin and rat serum were prepared according to the general plan of Goettsch and Kendall.<sup>4</sup> Each female Belgian rabbit received 1.0 mg of antigen intravenously in a volume of 0.1 cc, 3 times a week for 6 weeks. The rabbits were then exsanguinated and the active antisera against a given antigen were pooled and stored in the frozen state. Small portions for current use were stored at 0-2°C.

For the precipitin test, 1 cc of antiserum was added to 2 cc antigen diluted with 0.85% sodium chloride. Results were read after 2 hours at room temperature (25-28°C) followed by refrigeration at 0-2°C for 17 hours. There was no cross precipitation between the antisera and heterologous antigens.

For the quantitative determinations, known amounts of antigen protein measured by the gravimetric method of Barnett, Jones and Cohen,<sup>5</sup> were mixed with antiserum. The precipitates which formed in several tubes with a given antigen and antiserum were pooled and washed repeatedly with 0.85% sodium chloride solution at 0-2°C. Precipitation was performed in the range of marked antibody excess, determined by the addition of antigen to the supernatant fluid and re-incubation.

The washed precipitate was drained, then dissolved in a known volume of 4% sodium hydroxide. Ultraviolet light absorption of this solution was determined in a Beckmann spectrophotometer at 280 mμ.<sup>6</sup> For the latter purpose, serial dilutions were prepared with 4% sodium hydroxide.

<sup>4</sup> Goettsch, E., and Kendall, F. E., *J. Biol. Chem.*, 1935, **109**, 221.

<sup>5</sup> Barnett, C. W., Jones, R. B., and Cohn, R. B., *J. Exp. Med.*, 1932, **55**, 683.

<sup>6</sup> Use of this procedure was suggested by Dr. Erwin Haas.

<sup>6</sup> Hogness, T. R., and Potter, Van R., *Ann. Rev. Biochem.*, 1941, **10**, 509.

tered only in very low dilutions, and, quantitatively, the amounts of rat serum protein appearing (calculated by difference) are within the order of magnitude for normal protein excretion.

The data presented here cannot answer whether rat serum protein is excreted along with bovine albumin as the result of diminished tubular reabsorption or the result of increased glomerular permeability or filtration rate. Such information might be obtainable from further investigation under different conditions, varying the rates of protein excretion over a wider range. However, the data given do demonstrate a gross difference in the mechanism of proteinuria following administration of bovine albumin and egg albu-

min, proteins that differ widely in structure. This difference is consistent with differences found in the effect produced by intraperitoneal injections of these proteins upon hemoglobin excretion in the rat,<sup>2,3</sup> and is of special significance in that the data given here were obtained by an independent experimental approach.

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## 17252. Some Observations on Growth Factors Required by *Leuconostoc citrovorum*.\*

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From the Lederle Laboratories Division, American Cyanamid Company, Pearl River, N. Y.

The presence of a growth factor for *Leuconostoc citrovorum* S0S1 in various natural materials was reported by Sauberlich and Baumann.<sup>1</sup> They noted that the organism would respond to thymidine but it was concluded that some other active factor was present in a liver concentrate. The "citrovorum factor" in liver extract was differentiated<sup>2</sup> from a factor active for *Lactobacillus leichmannii* in liver extract by observing that the two factors migrated in opposite directions in an electric field. In the present report, further observations of the characteristics of the "citrovorum factor" are described.

**Experimental.** *Lactobacillus leichmannii* 315 (ATCC 7830) and *Leuconostoc citrovorum*

S0S1 were used in this study. Methods for the use of *Lactobacillus leichmannii* have been previously described;<sup>3-5</sup> the assay technic with *Leuconostoc citrovorum* followed that of Sauberlich and Baumann.<sup>1</sup>

The data of Table I indicate the response of the test organism to concentrated liver extract (15 U.S.P. units per cc), vitamin B<sub>12</sub> and thymidine before and after heating with alkali. *Lactobacillus leichmannii* gave heavy growth with only 0.05  $\mu$ l of untreated liver extract, but after treatment with alkali much higher levels of liver extract were required to promote good growth of the organism. As noted elsewhere this alkali treatment destroys the growth-promoting action of vitamin B<sub>12</sub>.

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<sup>1</sup> Sauberlich, H. E., and Baumann, C. A., *J. Biol. Chem.* 1945, **170**, 163.

<sup>2</sup> Lyman, C. M., and Prescott, J. M., *J. Biol. Chem.* 1949, **178**, 523.

<sup>3</sup> Snell, E. E., Rhee, E., and McNam, W. S., *J. Biol. Chem.* 1945, **175**, 473.

<sup>4</sup> Hoffmann, C. E., Stokstad, E. L. B., Franklin, A. L., and Jukes, T. H., *J. Biol. Chem.* 1948, **176**, 1465.

<sup>5</sup> Stokstad, E. L. B., Dornish, A. C., Franklin, A. L., Hoffmann, C. E., Howlings, B. L., and Jukes, T. H., *Fed. Proc.* 1949, **8**, 387.

TABLE III.  
Protein Excretion in Rats after Parenteral Bovine Albumin Injections.

Rat No.	Total prot. excretion, mg/24 hr	Total prot. excretion, mg/min.	Bovine alb. excretion, mg/min.	Ratio of bov. alb. to total prot. excreted
35	1440	1.000	0.738	0.74
53	750	0.521	0.369	0.71
61	859	0.597	0.366	0.62
62	842	0.585	0.408	0.70
80	320	0.222	0.167	0.75
45	634	0.441	0.245	0.56
47	918	0.638	0.351	0.55
71	884	0.614	0.398	0.65
74	737	0.512	0.293	0.57
98	1044	0.726	0.508	0.70
00	1160	0.806	0.534	0.66
Mean	872			$0.66 \pm 0.05$

TABLE IV.  
Protein Excretion in Rats after Parenteral Egg Albumin Injections.

Rat No.	Total prot. excretion, mg/24 hr	Total prot. excretion, mg/min.	Egg alb. excretion, mg/min.	Ratio of egg alb. to total prot. excreted
26	158	0.1101	0.1000	0.91
29	168	0.1163	0.1257	1.08
31	145	0.1009	0.0923	0.92
32	139	0.0967	0.0822	0.85
38	151	0.1048	0.1090	1.04
59	188	0.1303	0.1081	0.83
95	266	0.1847	0.1712	0.93
96	201	0.1399	0.0782	0.55
07	174	0.1210	0.0939	0.78
08	288	0.2002	0.1880	0.95
10	252	0.1750	0.1782	1.02
14	310	0.2153	0.2295	1.06
Mean	203			$0.91 \pm 0.10$

absorption measured. All determinations were performed in duplicate, and many in quadruplicate on more than one occasion. Total protein concentrations in the urine were determined by the biuret method.<sup>7</sup>

In studying the proteinuria following bovine albumin injections, we followed the same routine described by Addis and used in our previous work. Female rats weighing about 200 g received intraperitoneal injections of 6% bovine albumin in 0.85% sodium chloride solution. The injections, each of 16 cc, were given at 9:30 A.M., 4:30 P.M., and 9:30 A.M. on the next day. At the time of the last injection they were taken from stock diet and given 10% dextrose solution in 0.4% sodium chloride, in order to promote diuresis. Urine was collected from 10:00 A.M. to 3:00 P.M.

Because of differing dose-time relation-

ships, in the experiments with egg albumin it was necessary to use other conditions in order to collect urine at the height of proteinuria. Female rats of similar size were taken from stock diet at 3:30 P.M. and were given a single intraperitoneal injection of 6% egg albumin in 0.85% sodium chloride solution. These rats were then placed on the dextrose-salt solution diet and urine was collected until 9:30 A.M. the next morning. These rats appeared edematous but otherwise well, with large urine volumes.

*Results.* The results are given in Tables I to IV. It is readily seen that, after injections of bovine albumin, appreciable amounts of rat serum protein are also excreted. On the contrary, after injections of egg albumin, nearly all of the urinary protein is egg albumin, so that qualitatively weak positive reactions to rat serum antiserum are encoun-

<sup>7</sup> Kingsley, G. R., *J. Biol. Chem.*, 1939, **131**, 197.

tered only in very low dilutions, and, quantitatively, the amounts of rat serum protein appearing (calculated by difference) are within the order of magnitude for normal protein excretion.

The data presented here cannot answer whether rat serum protein is excreted along with bovine albumin as the result of diminished tubular reabsorption or the result of increased glomerular permeability or filtration rate. Such information might be obtainable from further investigation under different conditions, varying the rates of protein excretion over a wider range. However, the data given do demonstrate a gross difference in the mechanism of proteinuria following administration of bovine albumin and egg albu-

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<sup>2</sup> Lyman, C. M., and Prescott, J. M., *J. Biol. Chem.*, 1949, **178**, 523.

<sup>3</sup> Snell, E. E., Kitay, E., and McNutt, W. S., *J. Biol. Chem.*, 1948, **175**, 473.

<sup>4</sup> Hoffmann, C. E., Stokstad, E. L. R., Franklin, A. L., and Jukes, T. H., *J. Biol. Chem.*, 1948, **176**, 1465.

<sup>5</sup> Stokstad, E. L. R., Dornbush, A. C., Franklin, A. L., Hoffmann, C. E., Hutchings, B. L., and Jukes, T. H., *Fed. Proc.*, 1949, **8**, 257.

TABLE I.  
Effect of Alkali on Growth Promoting Action of Concentrated Liver Extract, 15 U.S.P. Units per cc, for *Lactobacillus leichmannii* and *Leuconostoc citrovorum*.

Additions to 2 ml medium	Optical density*			
	<i>Lactobacillus leichmannii</i>		<i>Leuconostoc citrovorum</i>	
	(a)	(b)	(a)	(b)
None	0.28	0.28	0.03	0.03
0.03 $\mu$ l liver extract	1.15	0.46	0.08	0.08
0.1 " " "	1.33	0.66	0.20	0.19
0.3 " " "	1.32	0.90	0.42	0.42
1.0 " " "	1.39	1.25	0.94	0.92
3.0 " " "	1.40	1.42	1.45	1.50
10.0 " " "	1.50	1.50	1.90	1.90
10 m $\gamma$ vitamin B <sub>12</sub>	1.40	0.40	0.03	0.03
10 $\gamma$ thymidine	1.20	1.00	0.32	0.34

\* Determined after 20 hr incubation.

(a) Untreated supplements.

(b) Supplements steamed for 30 min. with 0.2 N NaOH.

for *L. leichmannii*, although thymidine appears to be resistant.<sup>6</sup> The small residual effect of liver extract for *L. leichmannii* after alkali treatment is presumably due to thymidine and other desoxyribosides. In contrast to the results with *L. leichmannii*, the response of *Leuconostoc citrovorum* 8081 to liver extract was unchanged following treatment with alkali and it was also noted that *L. citrovorum* did not respond to vitamin B<sub>12</sub>. In accord with the results of Sauberlich and Baumann<sup>1</sup> it was found (Table I) that *L. citrovorum* gave only a partial growth response with thymidine as compared to a marked response with liver extract, thus supporting the view that there is an unknown "citrovorum factor", in addition to thymidine, in liver extract. The data of Table I demonstrate that this factor is not identical with the factor required by *Lactobacillus leichmannii*, and show that the "citrovorum factor" is stable to steaming with 0.2 N NaOH for 30 minutes. Lyman and Prescott<sup>7</sup> have noted that the "citrovorum factor" is stable to alkali.

The report that high levels of pteroylglutamic acid (PGA) could in the presence of purine bases replace the "citrovorum factor."<sup>8</sup> and the finding that 4-amino pteroylglutamic

acid, a PGA antagonist, produces an inhibition of the growth of *L. citrovorum* which is reversed by natural materials containing the "citrovorum factor,"<sup>8</sup> prompted further study of the role of PGA in the nutrition of *L. citrovorum*. The data of Table II show that although after 36 hours incubation thymidine or PGA when assayed singly can only partially replace the factor required by this organism, the simultaneous addition of thymidine and PGA to the medium resulted in marked growth after only 18 hours incubation. These results give additional indication of a relationship between PGA, thymidine and the "citrovorum factor." In other experiments, it was found that the addition of 10  $\gamma$  of vitamin B<sub>12</sub> per 2 ml medium to tubes containing thymidine and PGA had no additional effect on growth. The addition of *p*-aminobenzoic acid, pteroyltriglutamic acid, pteric acid or xanthopterin to tubes containing thymidine (Table II) in no instance gave the marked effect on growth produced by the mixture of thymidine and PGA. It was also noted that after a 12-hour incubation period the response of the organism to liver extract was much greater than the response to thymidine plus PGA. This finding suggests that the pre-formed factor was present in liver extract, but that in the tubes containing thymidine and PGA some additional transformation must have occurred. However, it is apparent that the presence of thymidine and PGA in natural materials could have a marked

<sup>6</sup> Hoffmann, C. E., Stokstad, E. L. R., Hutchings, B. L., Dornbush, A. C., and Jukes, T. H., *J. Biol. Chem.*, in press.

<sup>7</sup> Lyman, C. L., and Prescott, J. M., *Fed. Proc.*, 1949, 8, 220.

<sup>8</sup> Sauberlich, H. E., *Fed. Proc.*, 1949, 8, 247.

TABLE II.

Relationships Between Thymidine, Pteroylglutamic Acid, and Related Compounds for Growth of *Leuconostoc citrovorum*.

Additions to 2 ml medium	Optical density after incubation time of		
	12 hr	18 hr	36 hr
None	0.04	0.04	0.06
10 $\gamma$ thymidine	0.08	0.17	0.98
10 $\gamma$ pteroylglutamic acid (PGA)*	0.04	0.04	0.38
10 $\gamma$ thymidine + 10 $\gamma$ PGA	0.40	1.40	1.70
10 $\gamma$ thymidine + 10 $\gamma$ p-aminobenzoic acid	0.04	0.15	0.94
10 $\gamma$ " + 10 $\gamma$ pteroyltriglutamic acid	0.04	0.20	1.40
10 $\gamma$ " + 10 $\gamma$ pteric acid	0.04	0.15	1.02
10 $\gamma$ " + 10 $\gamma$ xanthopterin	0.04	0.15	0.96
10 $\mu$ l liver extract	1.10	1.80	1.80

\* Purified PGA containing at least 98.8% PGA (moisture free basis) was used in these experiments.

TABLE III.

Determination of  $R_f$  Values of Fractions of a Liver Extract, 15 Units per cc Separated by Paper Chromatography Using *Lactobacillus leichmannii* and *Leuconostoc citrovorum* as Indicators.

Substance chromatographed	Zones	$R_f$ values of growth factors	
		<i>Lactobacillus leichmannii</i>	<i>Leuconostoc citrovorum</i>
Liver extract	1	0.05	—
	2	0.45	—
	3	—	0.55
	4	0.60	0.64
	5	0.72	—
Vitamin B <sub>12</sub>	1	0.07	—
Thymidine	1	0.63	0.64
Mixture of guanine desoxy- riboside and hypoxanthine desoxyriboside	1	0.42	—

effect on the response of *L. citrovorum*.

Liver extract was separated into a number of fractions by paper-strip chromatography following a technic similar to that of Winsten and Eigen.<sup>9</sup> A mixture of 9 parts n-butanol: 1 part acetic acid was used as the mobile phase. The strip chromatograms were laid on a nutrient agar suitable for the growth of the test organisms, the agar was seeded with the appropriate organism, and incubated. The zones of growth indicated the positions of the growth factors present in the liver extract. Chromatograms of known compounds gave  $R_f$  values of aid in identifying the fractions separating in liver extract. Table III summarizes the  $R_f$  values found on chromatographing liver extract using *Lactobacillus*

*leichmannii* and *Leuconostoc citrovorum* as test organisms. In accord with recent investigations<sup>4,10,11</sup> *L. leichmannii* responded to fractions in liver corresponding to vitamin B<sub>12</sub> ( $R_f$  0.05), guanine and hypoxanthine desoxyribosides ( $R_f$  0.45), thymidine  $R_f$  0.60), and an as yet unidentified component ( $R_f$  0.72). Of great interest in the present investigation was the finding that *L. citrovorum* not only responded to thymidine ( $R_f$  0.64) but also to another component presumably the "citrovorum factor" ( $R_f$  0.55) in the liver extract. No zones of growth of *L. citrovorum* were observed in the positions on the chromatogram corresponding to vitamin

<sup>10</sup> Kitay, E., Snell, E. E., and McNutt, W. S., *J. Biol. Chem.*, 1949, **177**, 993.

<sup>11</sup> Hoff-Jorgensen, E., *J. Biol. Chem.*, 1949, **178**, 525.

<sup>9</sup> Winsten, W. A., and Eigen, E., *J. Biol. Chem.*, 1949, **177**, 989.

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<sup>7</sup> Lyman, C. L., and Prescott, J. M., *Fed. Proc.*, 1949, 8, 220.

<sup>8</sup> Sauberlich, H. E., *Fed. Proc.*, 1949, 8, 247.

paired ability for hepatic estrogenic inactivation".<sup>3</sup> In connection with the established lipotropic activity of estrogens in the strains hitherto tested<sup>1,2</sup> the claim that in the Fischer strain "extensive fatty infiltration of the liver develops when massive doses of estrogen are administered" (alpha-estradiol) appeared to be of particular interest. Thus, the possibility that the response of the inbred Fischer strain to estrogens might differ from that previously established in other strains, made it desirable to test the effect of estrogen on rats of this strain kept on an alipotropic diet.

*Experimental.* The same general procedures were followed as were previously reported.<sup>1,2</sup> Young adult rats, weighing 120-180 g, were used. All of the animals were bred and raised in our laboratory from rats of the Fischer strain received, through the courtesy of Dr. A. Segaloff, from Dr. W. F. Dunning (Wayne University, College of Medicine, Detroit). As usual, control and treated animals were run simultaneously in all experiments. When all of the animals of an experiment could not be run at one time, equal numbers of the various groups were started together. All of the animals received the same alipotropic diet which was of low protein-high fat type. It, and the vitamin supplements, were described in the previous paper.<sup>1</sup> In the experiments with ethinyl estradiol the hormone reduced the appetite of the rats to such an extent that it was necessary to run pair-fed controls.

As estrogens, estrone and ethinyl estradiol<sup>†</sup> were used, given orally,<sup>2</sup> the daily dose being dissolved in 2 drops of cottonseed oil. Control animals were given 2 drops of oil daily. When methionine was given, the daily dose (50 mg dl-methionine).<sup>‡</sup> was dissolved in 1 ml of water, and mixed with the vitamin B supplement. In certain of the experiments, where a number of the animals did not drink this mixture, it was given to all by stomach tube.

At the end of the 21 day experimental period, the animals were sacrificed and the liver analyzed for total lipide with the same

technique used in the previous experiments.

*Results and Discussion.* The results obtained are summarized in the attached Table. The most conspicuous difference in the reactivity of this inbred strain in comparison with that of other strains manifested itself in the very high fat content of the liver in the untreated animals fed the basal alipotropic diet. The average fat content of the liver both in male and in female controls of the inbred strain was found to be above 30% while with other strains in previous work<sup>1,2</sup> the corresponding figures were, as a rule, lower than 25%, and frequently less than 20%.

According to our observations made earlier, as well as to reports in the literature,<sup>4</sup> even within one experimental group the fat content of the liver shows often very considerable variations, with a distribution curve of wide extremes. In contrast, animals of the inbred Fischer strain of the present series showed remarkable uniformity of response. For instance, among 18 control animals of Experiments 21 and 22 (Table) only one rat showed a relatively low discrepant figure for liver fat, 14.6%, while the figures for all remaining 17 rats varied only between 30.8 and 43.8%, an exceptionally narrow range for this type of experiment.

Even with the use of the Fischer strain the lipotropic effect of estrogens could clearly be demonstrated, but only with ethinyl estradiol and not with the much weaker estrone, at least not with the doses of estrone given. Addition of methionine increased the lipotropic effect of ethinyl estradiol considerably but did not accentuate the effect of estrone. Even in genetically less homogeneous strains of rats estrone acted as a weak lipotropic agent or was found to be devoid of any lipotropic activity.<sup>1,2</sup> It is probable that in the highly inbred Fischer rats, kept on the basal alipotropic diet, the development of very intensive fat infiltration of the liver may exert a special inhibitory influence as to the manifestation of the lipotropic effect of estrogens. In further consequence, it is not surprising that

<sup>†</sup> Ethinyl estradiol was kindly supplied by Roche-Organon, Inc., Nutley, N. J.

<sup>‡</sup> Kindly furnished by Wyeth, Inc.

<sup>4</sup> Beveridge, J. M. R., Lucas, C. C., and O'Grady, M. K., *J. Biol. Chem.*, 1945, **160**, 505.



B<sub>12</sub> and the desoxyribosides of guanine and hypoxanthine. These observations are in full accord with direct growth experiments (Table I).<sup>10</sup> *L. leichmannii* showed no zone of growth on the chromatogram in the position where the "citrovorum factor" was located. Thus the data of Table III provide additional evidence for the separate identities of the factors required by *L. leichmannii* and *L. citrovorum* and demonstrate the existence in liver of an unknown component, not identical with thymidine, that promotes the growth of *L. citrovorum*.

Liver extract, treated with alkali as described in Table I gave results in a paper strip chromatogram identical with those shown in Table III except that vitamin B<sub>12</sub> was destroyed as indicated by lack of growth of *L. leichmannii* in the region appropriate to vitamin B<sub>12</sub>.

**Summary.** 1. *Leuconostoc citrovorum* was found to respond to a growth-promoting factor

in the concentrated liver extract, but this organism did not respond to vitamin B<sub>12</sub>. The alkali-stable nature of the "citrovorum factor" further contrasts it with vitamin B<sub>12</sub>. 2. Two fractions were separated from liver extract by paper strip chromatography; one of these fractions was presumably thymidine and promoted growth of *Lactobacillus leichmannii* and *Leuconostoc citrovorum*. The other fraction was inactive for *L. leichmannii* but active for *L. citrovorum*.

3. Although thymidine or high levels of pteroylglutamic acid (PGA) when tested singly were only partially effective in promoting growth of *L. citrovorum*, the simultaneous addition of thymidine plus PGA produced marked growth of the organism. This finding suggests a functional relationship between thymidine, PGA and the "citrovorum factor."

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## 17253. The Lipotropic Effect of Estrogenic Hormones in Inbred Rats.\*

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It has been shown<sup>1,2</sup> that estrogenic hormones exert in rats distinct lipotropic activity. In particular, estrogenic compounds (estrone, estradiol benzoate, ethinyl estradiol) allow a more efficient use of methionine as a lipotropic agent. In these studies, various possible sources of error were taken in consideration, such as difference in food intake, weight, sex and genetic identity. Further, it has been found necessary to run control and treated rats simultaneously in all experiments. This latter precautionary measure was promp-

ted by the observation that experimental groups when not run simultaneously often differed in their absolute response even under identical experimental conditions.

With the exception of a few experiments in which unidentified strains obtained from a local dealer were employed, the studies were carried out on rats of the Sprague-Dawley strain.<sup>1,2</sup> In view of the observation that various inbred strains may vary more than 500% in their ability to inactivate estrogen<sup>3</sup> it became necessary to extend the observations on the lipotropic activity of estrogens to other strains beyond those used in previous studies,<sup>1,2</sup> especially to inbred strains, such as the Fischer strain, which shows "both a high threshold for vaginal estrus and an im-

\* This work was supported by the Commission on Liver Disease of the Army Epidemiological Board, Preventive Medicine Division, Office of the Surgeon General, Washington, D.C.

<sup>1</sup> György, P., Rose, C. S., and Shipley, R. A. *Arch. Biochem.*, 1947, **12**, 125.

<sup>2</sup> György, P., Rose, C. S., and Shipley, R. A. *Arch. Biochem.*, 1949, **22**, 108.

<sup>3</sup> Segaloff, A., and Dunning, W. P., *Endocrinology*, 1946 **30**, 289.

the lipotropic effect of ethinyl estradiol *plus* methionine appeared to be more pronounced in Fischer rats than seen before<sup>1,2</sup> with rats of genetically different and less homogeneous strains. For instance, in one experiment (Table I, Exp. 24), the average fat content of the liver in the control animals was found to be 33.1%, in the group treated with methionine, 18.7%, whereas in rats treated with methionine *plus* ethinyl estradiol, the perfectly normal figure of 5.7% was reached. It should be kept in mind that in these experiments the animals were pair-fed.

In histological examinations<sup>3</sup> the testes of rats receiving estrogen showed signs of degeneration with consecutive atrophy. In the control groups the testes exhibited essentially normal histological findings. In female rats receiving estrogens constant estrus was observed in contrast to the control rats and to the animals treated with methionine only, which showed essentially normal estrous

<sup>3</sup> We are indebted to Dr. Harry Goldblatt (Institute for Medical Research, Cedars of Lebanon Hospital, Los Angeles, Calif.) for the histological examination of the testes.

cycles.

The exceptionally marked response of rats of the Fischer strain to an alipotropic diet is in good accord with the recently published behavior of the same strain with regard to methionine uptake by liver slices.<sup>5</sup> Compared with an inbred Wistar strain, liver slices obtained from Fischer rats showed a very low uptake of methionine. These observations together with the results here presented should reemphasize the importance of genetic variations in stock laboratory animals when used in this type of experiment.

**Conclusions.** 1. The fat infiltration of the liver in response to an alipotropic diet is more uniform and more intensive in rats of the inbred Fischer strain than that seen in the past in genetically less homogeneous strains. 2. Estrogen, in particular ethinyl estradiol, when given in combination with methionine, exerts a very marked lipotropic effect with a corresponding reduction of the liver fat to normal values.

<sup>5</sup> Rutman, R., Dempster, E., and Tarver, H., *J. Biol. Chem.*, 1949, **177**, 491.

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## 17254. Effects of Pituitary Adrenocorticotrophic Hormone (ACTH) in Children with Non-Addisonian Hypoglycemia.

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Severe chronic hypoglycemia of the non-Addisonian type presents a difficult etiologic and therapeutic problem, unless by good fortune it is found on direct examination of the pancreas to be due to a removable adenoma of the islet cells in that organ, a comparatively rare pathological condition. Dietary control of the blood sugar level, which may be quite successful in some cases of mild "functional" or "recurrent" hypoglycemia, is ineffectual in the severe persistent type. Surgical removal of as much as 80 to 85% of the histologically normal pancreas, a therapeutic procedure of last resort, is likely to give temporary relief only, as in two members of the series of pa-

tients included in the present investigation. Cautious use of alloxan in one severe case of idiopathic hypoglycemia appeared to be successful.<sup>1</sup> Despite its hepatotoxic action, this agent deserves further trial but only under the strictest supervision.

Repeated attempts on our part to force the gluconeogenetic action of cortin to a degree sufficient to control non-Addisonian hypoglycemia, while mildly encouraging in a few instances, have been unsuccessful in the main. However, the isolation of pituitary adreno-

<sup>1</sup> Talbot, N. B., Crawford, J. D., and Bailey, C. C., *Pediatrics*, 1948, **1**, 337.

TABLE I.  
Liver Fat Experiments on Fischer Rats.

Exp. No.	Date	Group	No. of rats	Sex	Treatment (daily)	Liver		Rat weight		Food intake, g/day
						Wt (g)	Total fat (%)	Initial (g)	Change (%)	
21	10-17-47 to 11-18-47	a	8	M	—	10.6 ± 0.48	37.4 ± 1.5	164 (152-184)	—	7.7 ± 0.14
		b	9	M	Methionine (50 mg)	9.4 ± 0.79	28.3 ± 2.3	166 (150-182)	—	7.6 ± 0.21
		c	9	M	Estrone (30 γ)	11.3 ± 0.55	38.6 ± 0.73	167 (153-182)	—	7.5 ± 0.21
		d	9	M	{ Methionine (50 mg) Estrone (30 γ)	8.4 ± 0.39	22.3 ± 2.5	163 (153-180)	—	7.1 ± 0.30
22	11 18-47 to 11-29-47	a	8	F	Methionine (50 mg)	6.9 ± 0.33	20.6 ± 2.4	158 (148-163)	—	6.0 ± 0.13
		b	8	F	{ Methionine (50 mg) Estrone (30 γ)	7.9 ± 0.69	23.5 ± 3.8	158 (147-170)	—	5.4 ± 0.17
24	6-12-48 to 6-25-48	a	10	M	—	7.8 ± 0.40	33.1 ± 2.3	164 (135-196)	—	4.1 ± 0.24
		b	10	M	Ethinyl estradiol (30 γ)	8.0 ± 0.33	26.0 ± 1.3	165 (140-182)	—	4.2 ± 0.21
25	8- 2-48 to 8-27-48	c	10	M	Methionine (50 mg)	6.0 ± 0.45	18.7 ± 2.9	149 (134-160)	—	4.2 ± 0.28
		d	10	M	{ Methionine (50 mg) Eth. est. (30 γ)	6.2 ± 0.22	5.7 ± 0.11	152 (139-165)	—	4.6 ± 0.11
		a	11	F	—	7.4 ± 1.3	31.5 ± 2.2	126 (121-152)	—	3.6 ± 0.34
		b	11	F	Eth. est. (30 γ)	7.4 ± 0.50	28.4 ± 1.7	132 (128-150)	—	3.7 ± 0.38
25	9-27-48 to 1-17-49	c	10	F	Methionine (50 mg)	6.1 ± 0.41	17.6 ± 2.2	140 (117-158)	—	4.5 ± 0.21
		d	10	F	{ Eth. est. (30 γ) Methionine (50 mg)	5.5 ± 0.22	8.9 ± 1.6	140 (122-160)	—	4.4 ± 0.18

In experiments 24 and 25, the rats were pair fed.

In experiments 24 and 25, the rats were pair fed.

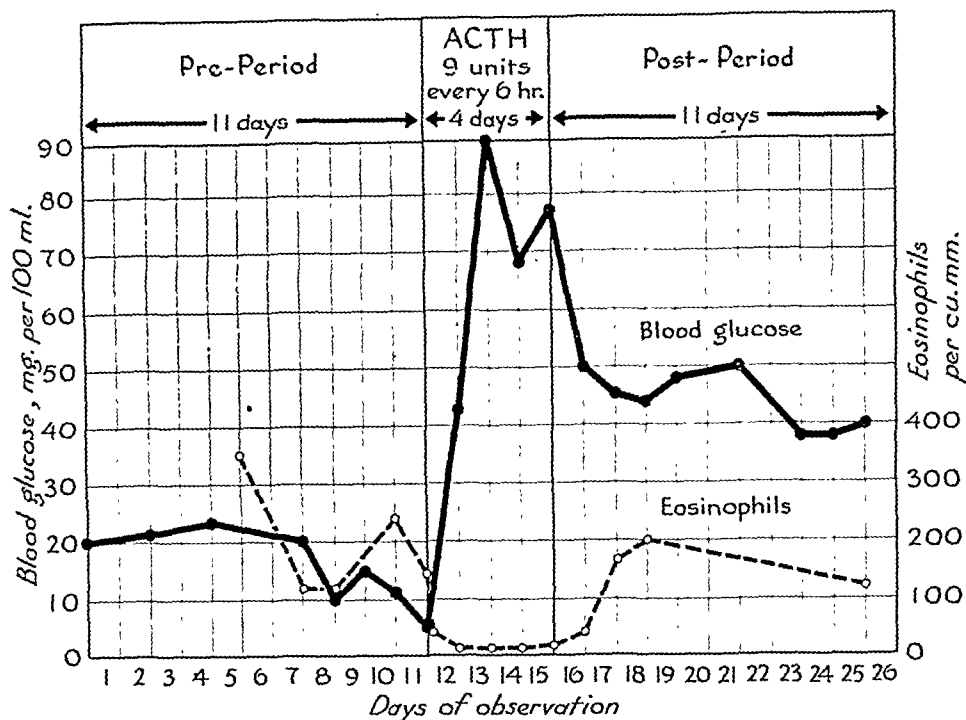


FIG. 1.

Effects of pituitary adrenocorticotrophic hormone (ACTH) on fasting blood glucose and eosinophil count in B.G.

period of similar length. Sugar tolerance tests were performed in the pre-period and again during the latter part of the ACTH period in all cases. In this test blood glucose was determined immediately before and fifteen minutes, 1 hour, 2 hours, 3 hours, 4 hours and 5 hours after termination of the intravenous infusion of a 25% solution of glucose (0.5 g glucose per kg of body wt.). The insulin sensitivity test was carried out by the "insulin-with-glucose" tolerance test proposed by Engel and Scott<sup>8</sup> on the last three patients studied. The ability of the pituitary gland to elaborate or to release ACTH was evaluated in these 3 patients by means of the test proposed by Recant, Forsham and Thorn,<sup>9</sup> according to which a marked decrease in the eosinophil count 4 hours following the

intravenous infusion of epinephrine indicates a normal response. The dosage of epinephrine was adjusted to the size of the child. R.R. was given 0.3 mg in 50 ml of saline solution; D.R. 0.4 mg in 60 ml, and Dn. R. 0.5 mg in 70 ml, the infusion running one hour.

**Results.** The experimental data on the 5 subjects with non-Addisonian hypoglycemia included in this study indicate that their responses to ACTH administration are similar in type to those reported for normal adult subjects.<sup>5,10</sup> Severe hypoglycemia and attendant symptoms were completely abolished during the period of intensive hormone administration and for at least a week after its withdrawal. The sharp fall and the abnormally prolonged low level of blood sugar in the glucose-tolerance curve, which represented the characteristic response of each of the patients during the control period, were

<sup>8</sup> Engel, F. E., and Scott, J. L., *Proc. Amer. Soc. Clin. Invest.*, Atlantic City, N. J., May 2, 1949, p. 17.

<sup>9</sup> Recant, L., Forsham, P. H., and Thorn, G. W., *J. Clin. Endocrin.*, 1948, 8, 589.

<sup>10</sup> Forsham, P. H., Thorn, G. W., Prunty, F. T. G., and Hills, A. G., *J. Clin. Endocrin.*, 1948, 8, 15.

corticotropin (ACTH) in purified form by Li, Evans and Simpson<sup>2</sup> and by Sayers, White and Long<sup>3</sup> and the demonstration of its diabetogenic effects in normal adult subjects by Browne<sup>4</sup> and Conn, Louis and Wheeler<sup>5</sup> encouraged us to resume our earlier attempts to counteract abnormal hypoglycemic reactions by the administration of a long-acting hormonal agent having a physiological action antagonistic to that of insulin.

The investigation on the metabolic and clinical effects of ACTH reported here was carried out first on 2 severely afflicted children (B.G., age 1 year, and her brother J. G., age 4½ years) and subsequently on 3 additional cases, (brothers, R.R., age 1 year, D.R., age 2½ years, and Derr. R., 4½ years of age) of the less severe "recurrent" or "functional" type. The infant, B.G., had manifested such frequent and severe hypoglycemic reactions, including numerous convulsions, that she had been subjected to partial pancreatectomy when the use of a variety of therapeutic measures, including frequent feedings of either a high-protein, low-carbohydrate, or a high-carbohydrate diet, had failed to alleviate her condition. An estimated 80 to 85% of her histologically normal pancreas had been extirpated. Although this radical procedure had resulted in restoration of the fasting blood sugar to normal values with complete relief from hypoglycemic symptoms for about three weeks, the hypoglycemic state had recurred shortly thereafter in a degree of severity almost as marked as that observed before the operation. The older brother, J.G., who had followed a similar metabolic and clinical course over a period of 3 years was only slightly better off than B.G., despite the surgical removal in two successive operations of even a greater proportion of his apparently normal pancreatic tissue. The other three patients had likewise had severe hypogly-

cemic symptoms including convulsions when for any reason their food intake was reduced.

*Methods.* In order to insure quantitative collections of all urine and feces as well as accurate accounting of the amounts of the special dietary formula consumed by each patient, special nurses were assigned to the small metabolic ward where the experimental subjects were kept on specially constructed collection frames. The standard semi-liquid diet, calculated to be nutritionally adequate in every respect, contained 42 g of protein, 45 g of fat and 118 g of carbohydrate in each kg. The daily allowance, adjusted to the caloric needs of the individual subject, was given in three equal meals shortly after 7:00 A.M., 1:00 P.M., and 7:00 P.M.

Fasting blood samples for glucose and eosinophil cell counts were obtained routinely at 7:00 A.M. every day or every second day throughout the entire period of study. The potassium and inorganic phosphorus of the serum were determined at the end of each major period. The 24-hour urine samples were analysed while fresh for total nitrogen, uric acid, creatinine, phosphorus, chloride, sodium and potassium throughout the entire period of study and for 11-oxy corticosteroids and 17-ketosteroids during the last two days of the control and ACTH periods. When successfully collected, stools for each major period were analysed for N, P, Cl, Na and K. Standard analytical methods were employed for the various substances named. The "true" blood glucose was determined by Nelson's<sup>6</sup> modification of the Shaffer-Somogyi<sup>7</sup> method, according to which values below 50 mg/100 ml are regarded as hypoglycemic.

In each experiment ACTH\* was administered intramuscularly in equal doses (either 9 or 10 mg equivalent to Armour standard preparation LA-1-A) every six hours over a period of 4 days. This test period was preceded by a control- or pre-period of several days length and was followed by a post-

<sup>2</sup> Li, C. H., Simpson, M. E., and Evans, H. M., *J. Biol. Chem.*, 1943, **149**, 413.

<sup>3</sup> Sayers, G., White, A., and Long, C. N. H., *J. Biol. Chem.*, 1943, **149**, 425.

<sup>4</sup> Browne, J. S. L., Josiah Macy Jr. Foundation Report, New York, June, 1943.

<sup>5</sup> Conn, J. W., Louis, L. H., and Wheeler, C. E., *J. Lab. Clin. Med.*, 1948, **33**, 651.

<sup>6</sup> Nelson, N., *J. Biol. Chem.*, 1944, **153**, 375.

<sup>7</sup> Shaffer, P. A., and Somogyi, M., *J. Biol. Chem.*, 1933, **100**, 695.

\* Supplied by the Armour Laboratories through the courtesy of Dr. John R. Mote, Medical Director.

the cases, were decreased from an average of 5.80 meq/l in the pre-period to 5.06 meq/l at the end of the ACTH period. At the same time the P was decreased from an average for the group of 3.03 to 2.79 meq/l. These decreases in serum K and P are interpreted as an indication of glycogen deposition during the ACTH period rather than being due to increased excretion. The magnitude of the decrease in retention of these two elements appeared to be far too small to influence blood composition.

The mucoprotein and the non-specific hyaluronidase inhibitor of the serums of the patients were both found by our colleagues, R.A. Good, V. C. Kelley, and D. Glick, to be increased at the end of the ACTH period by nearly 100% of the control levels. These elevations were transient, however, essentially normal values being found 4 days after withdrawal of the hormone.

**Summary.** The effects of ACTH on the fasting blood sugar level and glucose tolerance test; on the potassium and inorganic phosphorus content of the serum; on the nitrogen, phosphorus, chloride, sodium and potassium balances; on the urinary excretion of uric acid, creatinine and adrenal corticosteroids and on

the blood eosinophil counts were determined in 5 young children with non-Addisonian (familial) hypoglycemia. The type of response to ACTH was similar in all respects to that of the normal adult. However, under the conditions of this experiment, instead of producing a transient state of diabetes mellitus, as it does in the normal subject, the ACTH appeared merely to reverse the hypoglycemic tendency, with return of the fasting blood sugar level and the glucose tolerance curve to normal. While the eosinophil count returned to normal promptly upon withdrawal of ACTH, the blood sugar remained above the threshold for hypoglycemic reactions for at least 10 days without ACTH in the most severe case in the series (Fig. 1). Administration of 18 mg of ACTH in one dose every 48 hours thereafter served to maintain this one-year-old patient in an essentially non-hypoglycemic state for more than three additional weeks. Results of the study suggest that ACTH may prove to be as effective in the control of this non-Addisonian hypoglycemic disorder as insulin is in the control of diabetes mellitus.

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# 17255. Prevention of Chemotherapeutic Effects of 4-Amino-N<sup>10</sup>-Methyl-Pteroylglutamic Acid on Mouse Leukemia by Pteroylglutamic Acid.\*

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Certain derivatives of pteroylglutamic acid (PGA) which have in common the substitution of an amino group in the 4 position of the

pteridine ring have a demonstrable effect against some strains of transplanted mouse leukemia<sup>1-3</sup> and against solid tumors in mice

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<sup>†</sup> Fellow of The American Cancer Society, recommended by the Committee on Growth of The National Research Council.

<sup>1</sup> Burchenal, J. H., Burchenal, J. R., Kushida, M. N., Johnston, S. F., and Williams, B. S., *Cancer*, 1949, 2, 113.

<sup>2</sup> Law, L. W., *J. Nat. Can. Inst.*, in press.

<sup>3</sup> Burchenal, J. H., Johnston, S. F., Burchenal, J. R., Kushida, M. N., Robinson, E., and Stock, C. Chester, *Proc. Soc. Exp. Biol. and Med.*, 1949, 71, 381.

found to be absent during the period of ACTH administration, the curve becoming essentially normal. None of the 5 patients showed sugar in the urine at any time during the entire period of study, except for the mild glycosuria which occurred at the height of the glucose tolerance test made in the ACTH period. Insulin hypersensitivity was likewise counteracted to a large extent by the ACTH in the cases tested. The eosinophil cell counts 4 hours after infusion of epinephrine averaged 31/cmm compared to the average initial count of 133. This 79% fall was interpreted as indicating normal capacity on the part of the pituitary to produce ACTH in the 3 cases tested.

The eosinophil cells of the blood fell precipitately from the normal range (between 100 and 350/cmm) to between 0 and 10/cmm within 4 hours after ACTH was first administered and remained at this low level so long as the hormone was given at 6-hour intervals. While the fasting blood sugar during the post-period tended to remain for a number of days at levels intermediate between the hypoglycemic values of the pre-period and the normal values of the ACTH period, the eosinophils returned to their original normal counts fairly promptly, usually within a day. Responses of the blood glucose and eosinophil cell count to ACTH administration are illustrated in Fig. 1 which presents data obtained on B.G., the most severe case in the series. Changes in the other four cases were very similar to these. The only untoward effects of the ACTH were a transient vasopressor reaction after each injection and a moderate tendency to oliguria during the first day or two of intensive administration. These effects appear to be due to contamination with posterior pituitary hormones.

During a follow-up period of 94 days subsequent to the "post-period" shown in Fig. 1, ACTH was administered to B.G. in a dose of 18 mg once every 2 days. The fasting blood sugar was then determined at the end of each 48-hour period, when it would presumably be at its lowest level. The values so obtained were found to range between 40 and 68 mg/100 ml throughout the period, except for a few days when the patient refused

part of her diet because of an upper respiratory infection. Two of the morning values at that time were 24 and 16. Her diet, which was unrestricted, was taken avidly throughout the remainder of the period and the gain in weight was satisfactory. She learned to walk alone in the interval. No clinical signs of hypoglycemia or of toxic side effects were observed at any time. On the contrary, the ACTH appeared to be almost as specific for the control of this severe hypoglycemic disorder as insulin is for the control of diabetes mellitus.

The urinary excretion of 11-oxycorticosteroids and 17-ketosteroids was increased as a result of the ACTH injections by percentages ranging between 75 and 400. At the same time, the uric acid excretion increased 50 to 100%. Whereas intensive administration of ACTH has been reported to induce a negative nitrogen balance in most normal adult subjects, a positive balance was maintained in all periods in these very young, growing subjects, as long as the full diet was taken. However, the magnitude of the positive balance was less during the period of intensive ACTH administration than during the pre- and post-periods. In the one patient, B.G., whose stool, as well as urine, analyses have been completed up to date, the phosphorus balance was likewise slightly positive in all periods. The size of the positive balance during the ACTH period, however, was less than that for the fore-period and the after-period. Sodium and chloride showed no significant tendency to increased retention during the ACTH period, but showed a fairly marked increase in excretion in the post-period. The comparatively low NaCl content of the diet may account in part for the low degree of retention. Potassium showed a small negative balance during the ACTH period but positive balances for the pre-period and the post-period.

There were small but consistent changes in the potassium and inorganic phosphorus of the blood serum of all 5 of the subjects as a result of ACTH administration. In every instance both elements fell to lower levels. The fasting K values, which were slightly elevated in 3 and near the upper limit of normal in 2 of

TABLE I.  
Prevention of the Anti-leukemic Effect of 4-Amino-N<sup>10</sup>-Methyl-PGA by Pteroylglutamic Acid.

Experiment	Dose, mg/kg		No. of mice	Survival time in days		
	SK 1275	PGA		Range	Mean	S.D.
1	—	—	20	10-18	12	±1.87
	3	60	10	13-24	16	±3.32
	3	—	7	22-41	30	±5.84
2	—	—	18	10-18	13	±1.79
	3	30	20	12-29	17	±1.27
	3	—	19	27-49*	31	±5.3
3	—	—	18	11-17	13	±1.52
	3	60 × 4	17	9-18	16	±2.19
	3	30 × 4	—	—	—	—
4	—	—	11	21-59	30	±9.8
	3	60	22	9-14	11	±1.22
	3	30	10	12-17	14	±1.51
	3	15	10	10-19	14	±2.86
	3	3	10	12-43†	22	±8.48
	3	—	10	23-28	26	±1.84
	3	—	20	22-43†	30	±4.17

\* 2 mice surviving at 49 days.

† 1 mouse surviving at 43 days.

TABLE II.  
Prevention of Anti-leukemic Effect of 4-Amino-N<sup>10</sup>-Methyl-PGA by Pteroyltriglutamic Acid.

4-Amino-N <sup>10</sup> -methyl-PGA	Dose, mg/kg		No. of mice	Survival time in days		
	PTGA			Range	Mean	S.D.
—	—	—	20	8-13	10	±1.53
—	400	—	10	9-12	11	±1.38
3	400	—	9	12-19	16	±2.64
3	200	—	10	15-20	18	±1.69
3	100	—	9	13-24	19	±2.82
3	50	—	10	13-34*	20	±5.49
3	25	—	10	17-30	25	±3.89
3	12.5	—	10	19-30	26	±3.29
3	6.25	—	9	27-34*	29	±2.23
3	—	—	19	19-34†	28	±3.79

\* 1 mouse surviving at 34 days.

† 2 mice surviving at 34 days.

acid (PTGA)<sup>9</sup> was also tested by this technic for its ability to prevent the anti-leukemic effect of 4-amino-N<sup>10</sup>-methyl-PGA. Table II shows the effect of this conjugate of PGA. Some prevention of the chemotherapeutic action of a standard course of the antagonist was noted at 50 to 400 mg/kg, but none at 6.25 to 25 mg/kg.

**Discussion.** 4-amino-N<sup>10</sup>-methyl-PGA<sup>8</sup> was used in preference to 4-amino-PGA in these

<sup>9</sup> Boothe, J. H., Mowat, J. H., Hutchings, B. L., Angier, R. B., Waller, C. W., Stokstad, E. L. R., Semb, J., Gascola, A. L., and Subbarow, Y., *Trans. N. Y. Acad. Sc.*, 1948, 10, 70.

experiments since its chemotherapeutic effect against leukemia Ak 4 is greater and more consistent.<sup>1,3</sup> The inhibitory action of this anti-metabolite against *Streptococcus fecalis* R has been shown by Franklin *et al.*<sup>10</sup> to be of high degree, but reversible by approximately equal amounts of PGA over a range of 10 to 10,000  $\gamma$  per 10 ml of medium. In the weanling Wistar rat on a purified diet supplemented with 10.0 mg/kg of PGA, good growth occurred at 1.0 mg/kg of 4-amino-N<sup>10</sup>-methyl-PGA, but animals fed 3.0 mg of

<sup>10</sup> Franklin, A. L., Belt, M., Stokstad, E. L. R., and Jukes, T. H., *J. Biol. Chem.*, 1949, 177, 621.



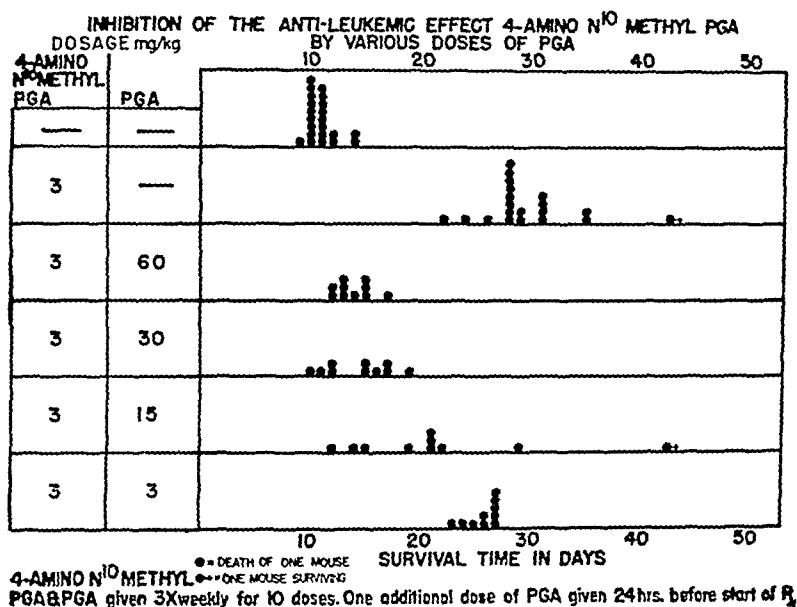


Fig. 1.

and rats.<sup>4-6</sup> The beneficial effects of such compounds in some cases of acute leukemia in children were first reported by Farber *et al.*<sup>7</sup> and have since been confirmed by numerous clinical trials.

In an attempt to elucidate the mode of action of the 4-amino analogs of PGA against leukemic cells, an investigation was undertaken to determine whether prior administration of PGA could prevent the anti-leukemic effects of 4-amino-N<sup>10</sup>-methyl-PGA.

**Experimental.** The procedure for inoculating mice of the AKm stock with leukemia Ak 4 was similar to that reported previously.<sup>1,3</sup> 0.1 ml of a saline suspension of leukemic spleen containing 1,000,000 cells was injected intraperitoneally. Forty-eight hours later,

treatment with 4-amino-N<sup>10</sup>-methyl-PGA<sup>4,8</sup> was started in the maximum tolerated dose of 3 mg/kg 3 times weekly for 10 doses by the intraperitoneal route. Intraperitoneal injections of PGA were started 24 hours after the inoculation of the leukemia and then given one hour before each dose of the antime-tabolite. Various dosage levels of PGA were studied against the standard dose of 4-amino-N<sup>10</sup>-methyl-PGA. As can be seen from Fig. 1 and Table I, PGA administered at levels of 60 and 30 mg/kg by this schedule blocked the anti-leukemic effects of the antagonist. The mice so treated died at approximately the same time as the controls whereas the mice treated with 4-amino-N<sup>10</sup>-methyl-PGA alone survived at least twice as long as the controls. When the dosage level of PGA was only 15 mg/kg, less blocking of effect was seen and with 3 mg/kg there was almost none.

A less toxic conjugate, pteroyltriglutamic

<sup>4</sup> Schoenbach, E. B., Goldin, A., Goldberg, B., and Ortega, L. G., *Cancer*, 1949, **2**, 57.

<sup>5</sup> Sugiura, K., Moore, A. D., and Stock, C. Chester, *Cancer*, 1949, **2**, 491.

<sup>6</sup> Moore, A. D., Stock, C. Chester, Sugiura, K., and Rhoads, C. P., *Proc. Soc. Exp. Biol. and Med.*, 1949, **70**, 396.

<sup>7</sup> Farber, S., Diamond, L. K., Mercer, R. D., Sylvester, R. F., and Wolff, J. A., *New Eng. J. Med.*, 1949, **238**, 787.

<sup>8</sup> We are indebted to Dr. Williams and the late Dr. Subarrow of the Lederle Laboratories for our supply of this compound.

<sup>8</sup> Smith, J. M., Jr., Cosulich, D. B., Hultquist, M. D., and Seeger, D. R., *Trans. N. Y. Acad. of Sc.*, 1948, **82**, 10.

TABLE I.

Prevention of the Anti-leukemic Effect of 4-Amino-N<sup>10</sup>-Methyl-PGA by Pteroylglutamic Acid.

Experiment	Dose, mg/kg		No. of mice	Survival time in days		
	SK 1275	PGA		Range	Mean	S.D.
1	—	—	20	10-18	12	±1.87
	3	60	10	13-24	16	±3.32
	3	—	7	22-41	30	±5.84
2	—	—	18	10-18	13	±1.79
	3	30	20	12-29	17	±1.27
	3	—	19	27-49*	31	±5.3
3	—	—	18	11-17	13	±1.52
	3	60 × 4	17	9-18	16	±2.19
	3	30 × 4	11	21-59	30	±9.8
4	—	—	22	9-14	11	±1.22
	3	60	10	12-17	14	±1.51
	3	30	10	10-19	14	±2.86
	3	15	10	12-43†	22	±8.48
	3	3	10	23-28	26	±1.84
	3	—	20	22-43†	30	±4.17

\* 2 mice surviving at 49 days.

† 1 mouse surviving at 43 days.

TABLE II.

Prevention of Anti-leukemic Effect of 4-Amino-N<sup>10</sup>-Methyl-PGA by Pteroyltriglutamic Acid.

Dose, mg/kg		No. of mice	Survival time in days		
4-Amino-N <sup>10</sup> -methyl-PGA	PTGA		Range	Mean	S.D.
—	—	20	8-13	10	±1.53
—	400	10	9-12	11	±1.38
3	400	9	12-19	16	±2.64
3	200	10	15-20	18	±1.69
3	100	9	13-24	19	±2.82
3	50	10	13-34*	20	±5.49
3	25	10	17-30	25	±3.89
3	12.5	10	19-30	26	±3.29
3	6.25	9	27-34*	29	±2.23
3	—	19	19-34†	28	±3.79

\* 1 mouse surviving at 34 days.

† 2 mice surviving at 34 days.

acid (PTGA)<sup>9</sup> was also tested by this technic for its ability to prevent the anti-leukemic effect of 4-amino-N<sup>10</sup>-methyl-PGA. Table II shows the effect of this conjugate of PGA. Some prevention of the chemotherapeutic action of a standard course of the antagonist was noted at 50 to 400 mg/kg, but none at 6.25 to 25 mg/kg.

**Discussion.** 4-amino-N<sup>10</sup>-methyl-PGA<sup>8</sup> was used in preference to 4-amino-PGA in these

<sup>9</sup> Boothe, J. H., Mowat, J. H., Hutchings, B. L., Angier, R. B., Waller, C. W., Stokstad, E. L. R., Semb, J., Gascola, A. L., and Subbarow, Y., *Trans. N. Y. Acad. Sc.*, 1948, 10, 70.

experiments since its chemotherapeutic effect against leukemia Ak 4 is greater and more consistent.<sup>1,3</sup> The inhibitory action of this anti-metabolite against *Streptococcus jecalis* R has been shown by Franklin *et al.*<sup>10</sup> to be of high degree, but reversible by approximately equal amounts of PGA over a range of 10 to 10,000  $\gamma$  per 10 ml of medium. In the weanling Wistar rat on a purified diet supplemented with 10.0 mg/kg of PGA, good growth occurred at 1.0 mg/kg of 4-amino-N<sup>10</sup>-methyl-PGA, but animals fed 3.0 mg of

<sup>10</sup> Franklin, A. L., Belt, M., Stokstad, E. L. R., and Jukes, T. H., *J. Biol. Chem.*, 1949, 177, 621.

the anti-metabolite per kilo of diet all died. When the dosage of PGA was increased to 100 mg/kg the rats at the 3.0 mg/kg level survived, but no protection was noted at the 10 mg/kg level of anti-metabolite. In the chick, the same investigators<sup>10</sup> found a slight inhibition of growth by 3 mg of the antagonist per kilo of purified diet in the presence of 0.1 mg/kg of PGA. When the level of PGA was raised to 10 mg/kg, this inhibition of growth was prevented. Hertz and Tullner<sup>11</sup> found that with this compound inhibition of the estrogen induced response of the chick oviduct could be obtained by 3 daily subcutaneous injections of 0.2 mg each. Blocking of this inhibition was demonstrated with 3 daily doses of 5 mg each of PGA given subcutaneously one hour prior to the injection of the anti-metabolite.

With a closely related antagonist, 4-amino-PGA, the prevention by PGA of toxicity,<sup>12,13</sup> of inhibition of estrogen induced growth of

uterus or oviduct,<sup>11</sup> and of the inhibitory effect against mouse leukemia<sup>2</sup> and solid tumors<sup>4</sup> has been observed.

It has been shown above that prevention of the toxic effects of these 2 analogs of PGA may be obtained within narrow ranges at the minimum toxic dose by high dosage of PGA. Since the dose of 4-amino-N<sup>10</sup>-methyl-PGA used in these experiments was just below the toxic dose, it was to be expected that an effect obtained at this level might be counteracted by PGA. The ratio of PGA to anti-metabolite necessary to prevent this anti-leukemic effect was approximately similar to that required in other studies.<sup>10,11</sup> The ability of PGA and PTGA to prevent the anti-leukemic effects of this anti-metabolite would seem to lend support to the theory that the chemotherapeutic effect of this derivative is due to its action as a metabolic antagonist of PGA.

**Summary.** The effect of 4-amino-N<sup>10</sup>-methyl-pteroylglutamic acid in prolonging the survival time of mice with transplanted leukemia Ak 4 can be blocked almost completely by prior administration of 10 to 20 times as much pteroylglutamic acid and, to a lesser degree, by 17 to 125 times as much pteroyltri-glutamic acid.

<sup>11</sup> Hertz, R., and Tullner, W. W., *Endocrinology*, 1949, **44**, 278.

<sup>12</sup> Franklin, A. L., Stokstad, E. L. R., and Jukes, T. H., *Proc. Soc. Exp. Biol. and Med.*, 1948, **67**, 398.

<sup>13</sup> Philips, F. S., and Thiersch, J. B., *J. Pharm. and Exp. Therap.*, 1949, **95**, 303.

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## 17256. Effect of Aluminum Hydroxide Gel and Calcium Lactate on Serum Bicarbonate.\*

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The observations herein reported indicate that subjects with reduced kidney function respond to the oral administration of suitable amounts of calcium lactate and aluminum hydroxide gel with a marked and sustained

elevation of serum bicarbonate. This response occurred both in very young infants with physiologically immature kidneys<sup>1</sup> and in older infants and children with kidney disease. No such increase in serum bicarbonate accompanied the administration of comparable amounts of the two agents to older infants and children with normal kidney function.

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<sup>1</sup> Barnett, H. L., Hare, K., McNamara, H., and Hare, R., *J. Clin. Invest.*, 1948, **27**, 691.

TABLE I.  
Effect of Aluminum Hydroxide Gel and Calcium Lactate on Serum Bicarbonate.

Subject	Age, days	Wt., kg	Daily oral intake		Before treatment	Serum bicarbonate content, <sup>a</sup> mN/L									
			5% aluminum hydroxide gel, ml/kg	Ca-lactate g/kg		During treatment,* days									
						1	2	3	4	5					
Young infants.															
OA	1	4.2	10	1.6	27.0	26.8	33.5								
OR	2	3.2	7.5	1.5	24.7	26.2	32.3	34.1							
AD	2	3.6	11	1.3	20.9	25.0	29.1	27.3	26.7						
FR	7	4.2	7	1.1	19.6		25.8	24.3	29.1						
JO	9	3.3	11	1.7	22.7	25.2	29.1	30.8							
RA	21	2.7	15	1.6	26.3	31.4	30.9	33.8							
NE	28	3.0	7	1.4	25.8	31.9	36.7								
Children with kidney disease.															
MA	Years 1	7.5 7.3	10.0 10.3	1.5 1.0	16.2 19.6	20.2 25.4	24.0 26.7	25.7 29.1	26.0 32.3						
GR	14	7.5 33.0	10.0 3.9	1.0 0.6	19.5 21.5		24.3 28.3	25.5							
Infants and children with normal kidney function.															
AN	16 mos.	10.2	8.5	1.3	25.7	25.9	25.4	26.5							
UR	23 mos.	6.2	9.0	1.4	25.3	27.3	27.3	25.2							
RO	5½ yrs.	16.0	8.8	1.2	26.8	30.6	27.2	29.2							
AR	8½ yrs.	16.0	9.4	1.3	30.6	30.4		31.5							

<sup>a</sup> The last value coincides with the final day of treatment with both drugs. Serum bicarbonate values returned to pre-treatment levels within 24 to 48 hours following discontinuation of either or both drugs.

<sup>a</sup> Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, 1924, **61**, 523.

The quantities of calcium lactate and aluminum hydroxide gel given and the magnitude and rate of change in serum bicarbonate are shown in Table I.

Given separately, neither agent produced an increase in serum bicarbonate in subjects with either normal or reduced kidney function. Failure of aluminum hydroxide gel to raise serum bicarbonate in subjects with normal kidney function is in accord with earlier reports.<sup>2</sup> Its effect in subjects with reduced kidney function is less well established. Freeman and Freeman<sup>3</sup> gave this drug to children with chronic nephritis but did not report detailed data on serum bicarbonate. They did mention relief from acidosis a few days after adding aluminum hydroxide gel to the low phosphorus diet of a 10 year old boy with chronic renal insufficiency.<sup>4</sup> From our findings, correction of renal acidosis with aluminum hydroxide gel alone would not be expected. The apparent discrepancy may, perhaps, be explained by differences in phosphorus intake since our subjects received milk, and therefore relatively large amounts of phosphorus.

The mechanisms underlying the difference in response of serum bicarbonate in subjects with reduced and normal kidney function to calcium lactate and aluminum hydroxide gel are not yet explained. Detailed balance studies are now in progress in an attempt to

define these mechanisms.

The observed increase in serum bicarbonate in subjects with reduced kidney function following oral calcium lactate and aluminum hydroxide gel is of interest in several respects. It is known that aluminum hydroxide gel alone lowers serum phosphate and raises serum calcium in patients with chronic renal acidosis.<sup>5</sup> Our results indicate that calcium lactate given with the aluminum hydroxide gel may correct the acidosis. The possible application of the combined use of the two drugs in the treatment of tetany of the newborn is under investigation. Finally, the fact that elevation of serum bicarbonate occurred in very young infants suggests that the ability of the immature kidney of young infants to excrete excess base may be less well developed than its ability to conserve base.<sup>5</sup> Observations are planned to study this function in young infants directly.

**Conclusions.** The combined oral administration of calcium lactate and aluminum hydroxide gel produced a marked and sustained elevation of serum bicarbonate in young infants with physiologically low kidney function and in older subjects with impaired function. This effect did not occur in subjects with normal kidney function. Given separately, neither drug produced an increase in serum bicarbonate in subjects with normal or reduced kidney function.

<sup>2</sup> Kirsner, J. B., *Am. J. Digest. Dis.*, 1941, **8**, 160.

<sup>3</sup> Freeman, S., and Freeman, W. M. C., *Am. J. Dis. Child.*, 1941, **61**, 981.

<sup>4</sup> Freeman, S., and Freeman, W. M. C., *Quart. Bull. Northwestern U. Med. School*, 1943-45, **17-19**, 275.

We are indebted to Flora Hurwitz, R.N., for nursing and technical assistance.

<sup>5</sup> Gordon, H. H., McNamara, H., and Benjamin, H. R., *Pediatrics*, 1948, **2**, 291.

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# 17257. Effect of Febrile Plasma, Typhoid Vaccine and Nitrogen Mustard on Renal Manifestations of Human Glomerulonephritis.

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Remissions have been observed to occur spontaneously in patients with chronic diffuse glomerulonephritis in the nephrotic phase. Blumberg and Cassady<sup>1</sup> and Janeway<sup>2</sup> have reported that patients with diffuse glomerulonephritis after accidental or induced measles show transient reduction in protein excretion and significant diuresis, confirming the generally accepted opinion that remissions in this disease may be occasioned by intercurrent infection. Since the febrile phase of infection may be accompanied by profound disturbance in renal hemodynamics<sup>3,4</sup> it seemed possible that both decrease in proteinuria and diuresis might be directly attributable to alteration in renal hemodynamics.

On the other hand, Becker<sup>5</sup> has shown that administration of  $\text{HN}_2$  prevents the development of the Schwartzman phenomenon in

rabbits.<sup>†</sup> Since there is reason to believe that diffuse glomerulonephritis may be the result of immunological alteration in renal tissue, it seemed conceivable that a common factor might be operative in infection and following the administration of these agents. In this view it was conceivable that reactive factors might be present in the plasma of patients with acute infections which would produce remission of the nephrotic syndrome.

To test these possibilities, we have explored the effects of 1) pyrogenic reaction, 2) infusion of plasma from patients acutely ill with pneumococcal and hemolytic streptococcal infections, and 3) one of the nitrogen mustards, methyl bis(B-chloroethyl)amine hydrochloride ( $\text{HN}_2$ ), on protein excretion, diuresis, and rate of glomerular filtration in patients with diffuse glomerulonephritis.

**Methods.** Observations were made on daily weight, 24 hour urinary volume and urinary protein excretion, rate of glomerular filtration ( $C_{\text{IN}}$ ) as measured by the inulin clearance, and renal plasma flow ( $C_{\text{PAH}}$ ) as measured by the p-aminohippurate clearance in 4 patients with chronic diffuse glomerulonephritis in the nephrotic phase.

Urinary protein was determined by the micro-Kjeldahl and biuret methods<sup>8</sup> and inulin and p-aminohippurate by methods previously described.<sup>9</sup>

**Results.** The pyrogenic reaction was induced in one patient with marked renal functional impairment on 2 occasions with intravenously administered typhoid vaccine. On both occasions there was marked decrease in proteinuria, accompanied in the one instance in which it was measured by decreased filtra-

\* Assistant in Medicine and Fellow in Physiology. Aided in part by grants from the Commonwealth Fund and the New York Heart Association.

<sup>1</sup> Blumberg, R. W., and Cassady, H. A., *Am. J. Dis. Child.*, 1947, **73**, 151.

<sup>2</sup> Janeway, C. A., Moll, G. H., Armstrong, S. H., Jr., Wallace, W. M., Hallman, N., and Barnes, L. A., *Tr. A. Am. Physicians*, 1948, **61**, 108.

<sup>3</sup> Chasis, H., Ranges, H. A., Goldring, W., and Smith, H. W., *J. Clin. Invest.*, 1938, **17**, 683.

<sup>4</sup> Bradley, S. E., Chasis, H., Goldring, W., and Smith, H. W., *J. Clin. Invest.*, 1945, **24**, 749.

<sup>5</sup> Becker, R. M., *Proc. Soc. Exp. Biol. and Med.*, 1948, **60**, 247.

<sup>†</sup> Since our investigations were initiated, Janeway *et al.*<sup>6</sup> have reported that experimental nephritis produced in rabbits by the administration of bovine gamma globulin can be prevented by  $\text{HN}_2$  and Dammin and Bukantz<sup>7</sup> have shown that  $\text{HN}_2$  inhibits antibody production, Arthus reaction, and vascular lesions in rabbits produced by horse serum antigen.

<sup>6</sup> Janeway, C. A., Schwab, L., Moll, F. C., Hall, T., and Hawn, C. V., personal communication.

<sup>7</sup> Dammin, G. J., and Bukantz, S. C., *J.A.M.A.*, 1949, **139**, 358.

<sup>8</sup> Hiller, A., Greif, R. L., and Beckman, W. W., *J. Biol. Chem.*, 1948, **176**, 1421.

<sup>9</sup> Goldring, W., and Chasis, H., Hypertension and Hypertensive Disease, Commonwealth Fund, New York, 1944.

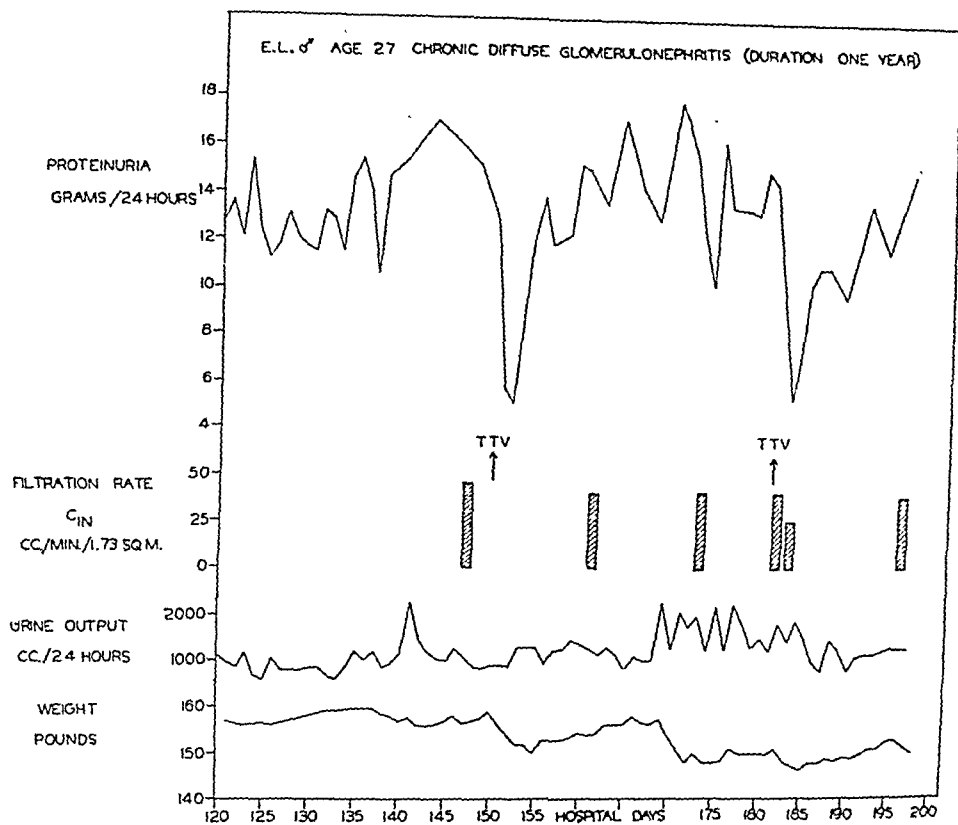


Fig. 1.

Effect of pyrogenic reaction on proteinuria and rate of glomerular filtration.

tion rate. No diuresis occurred (Fig. 1). It is our tentative opinion that the decrease in proteinuria was related to the renal hemodynamic alteration as manifested in part by decrease in filtration rate.

Two patients were given 1975 and 1280 cc of plasma intravenously respectively from patients with hemolytic streptococcal and pneumococcal infections in amounts of 125 to 500 cc at intervals in one patient over a period of one month and in the other over a period of 4 months. In neither patient was there a decrease in proteinuria or diuresis.

$\text{HN}_2$  was administered intravenously to 3 patients in 2 doses of 0.2 mg per kilo on successive days. In all 3 proteinuria decreased. In patient B.D. whose control  $C_{IN}$  and  $C_{PAH}$  were 13.9 cc and 66.3 cc per minute respectively, daily excretion of protein during a control period of 28 days ranged from 21.4

to 14.5 g, averaging 17.0 g. Protein excretion decreased to 2.3 and 10.6 g in the 24 to 48 and the 48 to 72 hour periods following the administration of  $\text{HN}_2$ , after which it returned to the pretreatment level. Diuresis did not occur. In patient L. G. whose control  $C_{IN}$  and  $C_{PAH}$  were 10.9 and 236 cc per minute, the daily excretion of protein during a control period of 113 days ranged from 9.1 to 4.2 g, averaging 7.0 g. Following administration of  $\text{HN}_2$  proteinuria decreased to 4.0 g per day, the decrease being first observed during the first 24 hours of  $\text{HN}_2$  administration, and persisting for 4 days. Diuresis did not occur.

Patient J.H., whose control  $C_{IN}$  and  $C_{PAH}$  were 116 and 839 cc per minute, was given  $\text{HN}_2$  on 2 separate occasions. As shown in Fig. 2, in each instance there occurred diuresis and marked decrease in protein-

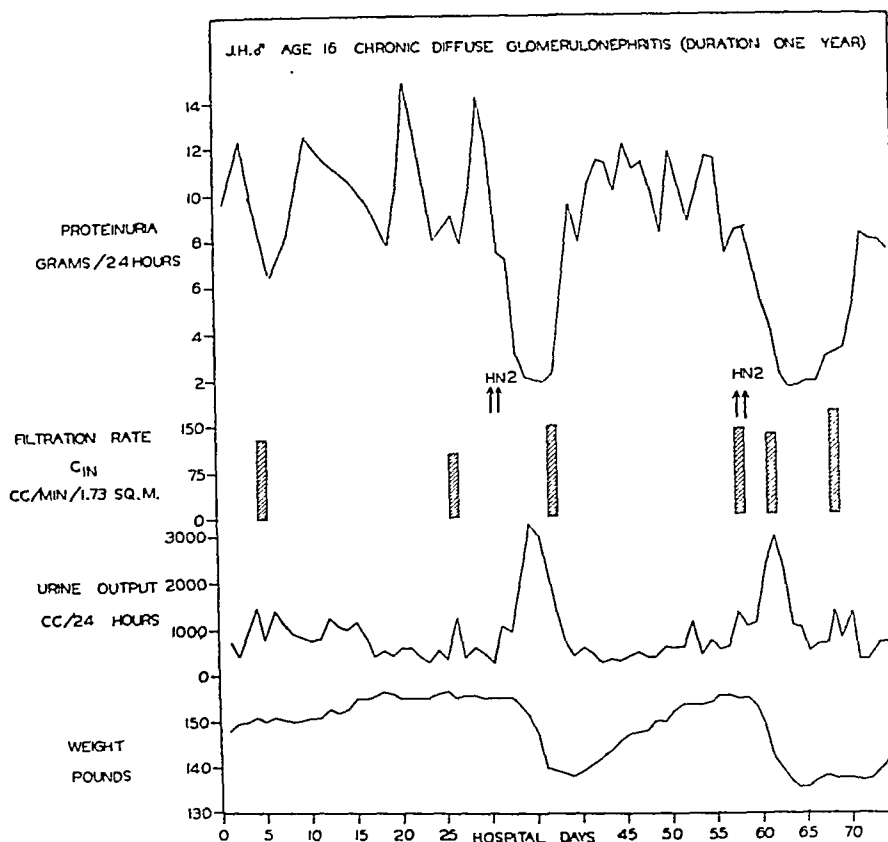


FIG. 2.

Effect of nitrogen mustard on proteinuria and rate of glomerular filtration.

uria, accompanied by an increased filtration rate. The diuresis was associated with disappearance of edema.

**Summary.** Administration of plasma from patients acutely ill with pneumococcal and streptococcal infections failed to decrease proteinuria or induce diuresis in 2 patients with chronic diffuse glomerulonephritis.

Induction of the pyrogenic reaction was accompanied by decrease in proteinuria on 2 occasions in one patient. We are inclined to attribute this result to the concomitant decrease in the rate of glomerular filtration.

Therapeutic doses of  $\text{HN}_2$  reduced proteinuria but diuresis failed to occur in 2 patients with advanced chronic diffuse glomerulonephritis with marked renal functional impairment. However, in one patient with minimal renal functional impairment, administra-

tion of  $\text{HN}_2$  was followed on 2 separate occasions by diuresis, marked reduction in proteinuria and concomitant increase in filtration rate, a combination of effects consistent with a return of glomerular function towards normal.<sup>‡</sup>

Our observations indicate that reversal of renal manifestations of human glomerulonephritis can be induced by  $\text{HN}_2$ .

This study is being extended to include patients in earlier phases of glomerulonephritis.

‡ It is of interest in this connection that in dogs the filtration rate may be substantially increased 40 to 72 hours after intravenous administration of  $\text{HN}_3$ .<sup>10</sup>

<sup>10</sup> Houck, C. R., Crawford, B., Bannon, J. H., and Smith, H. W., *J. Pharm. and Exp. Therap.*, 1947, **90**, 277.



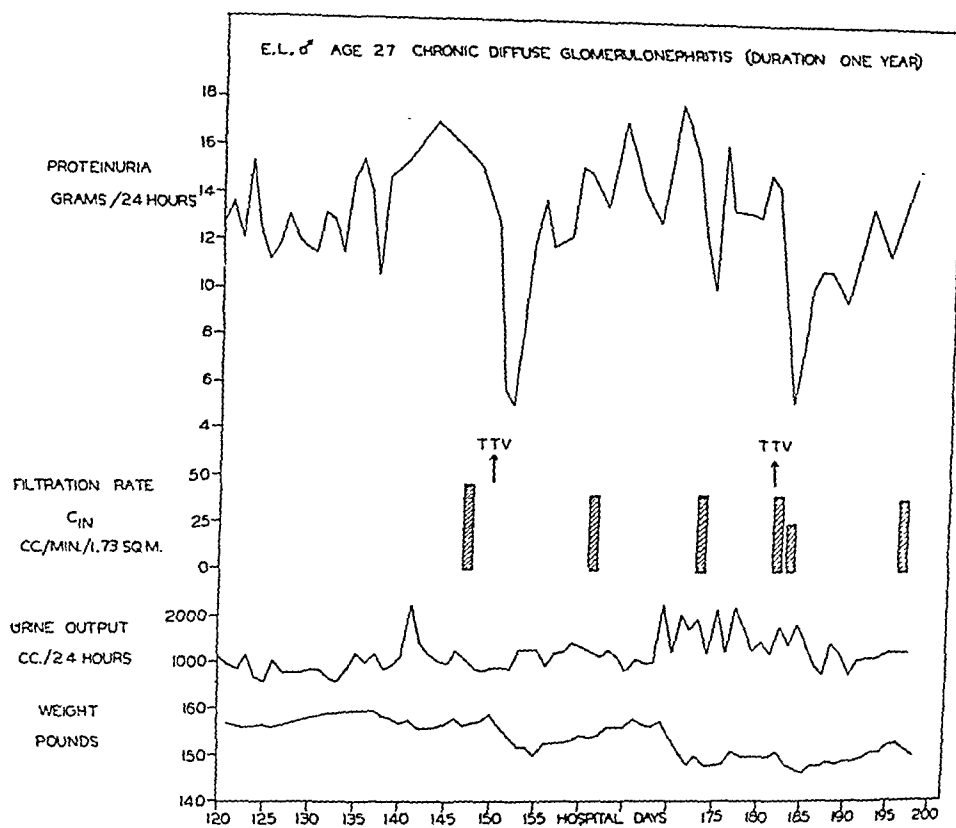


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Patient J.H., whose control  $C_{IN}$  and  $C_{PAH}$  were 116 and 839 cc per minute, was given  $HN_2$  on 2 separate occasions. As shown in Fig. 2, in each instance there occurred diuresis and marked decrease in protein-

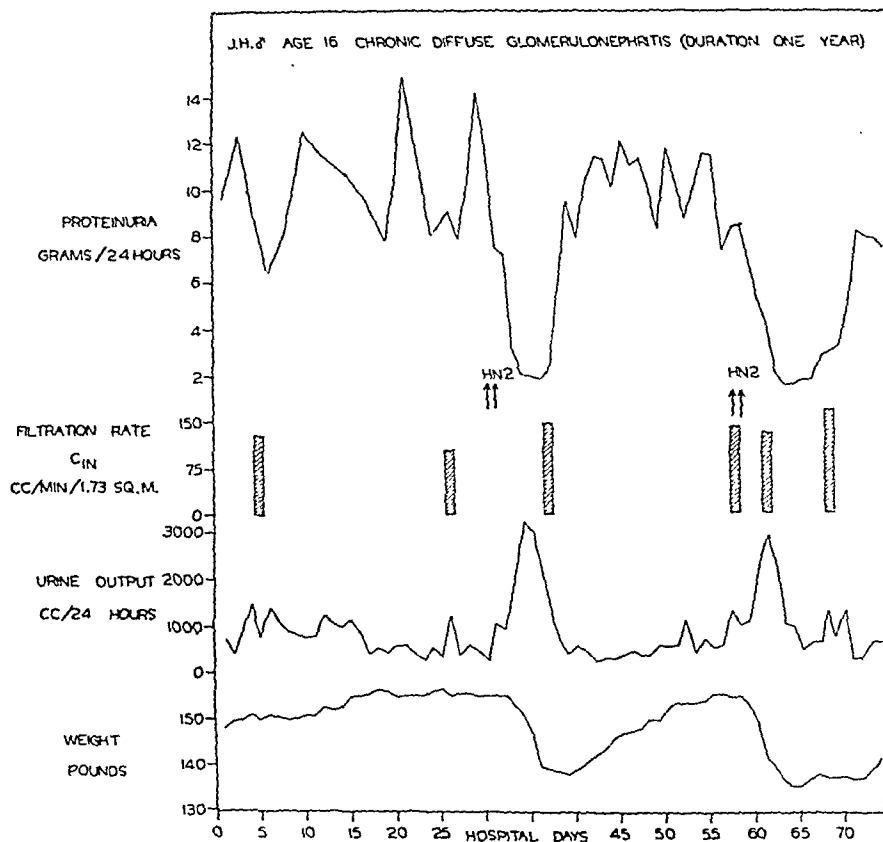


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tion of HN<sub>2</sub> was followed on 2 separate occasions by diuresis, marked reduction in proteinuria and concomitant increase in filtration rate, a combination of effects consistent with a return of glomerular function towards normal.<sup>‡</sup>

Our observations indicate that reversal of renal manifestations of human glomerulonephritis can be induced by HN<sub>2</sub>.

This study is being extended to include patients in earlier phases of glomerulonephritis.

<sup>‡</sup> It is of interest in this connection that in dogs the filtration rate may be substantially increased 40 to 72 hours after intravenous administration of HN<sub>2</sub>.<sup>10</sup>

<sup>10</sup> Houck, C. R., Crawford, B., Bannon, J. H., and Smith, H. W., *J. Pharm. and Exp. Therap.*, 1947, **90**, 277.

# 17258. Occurrence of a Transient Leucocytosis During the Jarisch-Herxheimer Reaction.\*

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There is no agreement in the medical literature on the characteristic blood picture in untreated early syphilis. Willcox<sup>1</sup> in a study of 405 patients with early syphilis found an average total white blood count of 8,950 with a normal differential. Previous to this report, findings of leucocytosis,<sup>2</sup> lymphocytosis,<sup>3</sup> monocytosis,<sup>4</sup> and eosinophilia<sup>5</sup> fill the literature on this subject.

No report exists, as far as can be determined, concerning the changes in the peripheral blood picture during a Jarisch-Herxheimer reaction. Sheldon and Heyman<sup>6,7</sup> have reported the occurrence of an acute polymorphonuclear leucocyte infiltration around early syphilitic lesions during a Jarisch-Herxheimer reaction, but they make no mention of the peripheral blood picture.

**Material.** Seventeen patients with early syphilis were used in this study. Of these 3 were seronegative primary, 6 were seropositive primary, and 8 were secondary. Four patients were female and 13 were male.

**Method.** After a diagnosis of syphilis was made and the pre-treatment white blood counts, differentials, and base line temperatures were completed, the patients were given a 300,000 unit dose of aqueous penicillin-G intramuscularly. They were then followed at

2 hourly intervals with total white blood counts, differentials and rectal temperatures. A total of 8 patients were followed with differential counts throughout the entire course of the reaction. All white counts were done by one observer, and 200 cells were counted in each differential. Periods of observation continued until the temperature returned to the pre-treatment level. The periods of observation varied from 10 to 16 hours in duration. At the end of the period of observation treatment was continued until a total of 2.4 million units of penicillin-G had been given over an 8-day period.

**Results.** Of the 17 patients, 15 had definite febrile Jarisch-Herxheimer reactions (defined here as in temperature to 37.5°C or above, occurring within 10 hours after the start of treatment and returning to normal within 16 to 18 hours). The distribution of the temperature elevations was as follows:

Temperature height	No. of patients.
37.8-38	3
38-39	3
39-40	8
40-41	1

All temperatures were rectal and 37.5°C was taken as normal temperature.

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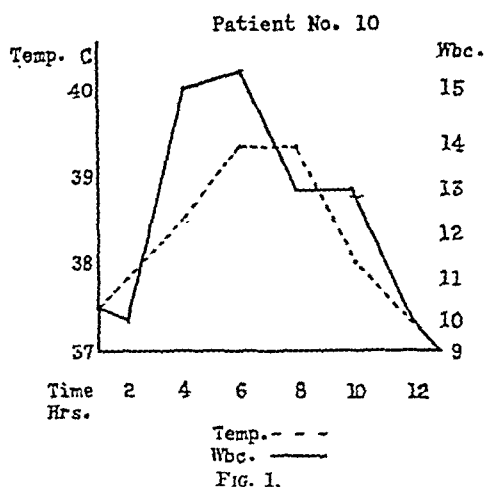
<sup>3</sup> Wintrobe, *Clinical Hematology*, Lea and Febiger, Philadelphia, 1942, p. 123.

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average duration being 4.4 hours. The longest period of duration was 8 hours, the shortest was 2 hours.

Fig. 1 indicates the type of response seen during the Jarisch-Herxheimer reaction.

Complete differential counts were done on 8 of the 15 patients every 2 hours during the Jarisch-Herxheimer reaction. The changes observed here were an increase in neutrophils with a lymphopenia. The average percentage increase in polymorphonuclear cells was 43.4% with a maximum change of 87% and a minimum change of 23%. The average percentage drop in lymphocytes was 55% with a maximum drop of 77% and a minimum drop of 39%. The maximum drop in lymphocytes occurred in the cases with the maximum increase in polymorphonuclear cells, and the minimum drop in lymphocytes occurred in the cases with the minimum increase in polymorphonuclear cells.

**Discussion.** The occurrence of the leucocytosis was at first felt to be caused by the fever associated with the Jarisch-Herxheimer reaction and not related to the mechanism of the reaction. The pattern of leucocyte re-

sponse found in induced fever was found to be quite different;<sup>8,9</sup> the rise in leucocytes in induced fever usually occurred after the height of the fever and the leucocytosis continued for several hours after a return to normal temperature. In this study the leucocytosis usually occurred 2 to 4 hours before the temperature spike and returned to the base line count at the same time or just before the temperature. It therefore appeared that the mechanism causing the leucocytosis was the same mechanism as that causing the Jarisch-Herxheimer reaction. The obscurity of the mechanism involved in the production of the Jarisch-Herxheimer reaction prevents any conclusions as to the causative factors. However, it is interesting to note that with the leucocytosis there is a neutrophilia which corresponds in time with the acute polymorphonuclear infiltration around syphilitic lesions as described by Sheldon and Heyman.<sup>6,7</sup>

**Summary.** 1. In 10 of 15 patients having febrile Jarisch-Herxheimer reactions there was a definite leucocytosis occurring before or at the same time as the temperature spike. Some degree of leucocytosis occurred in the other 5 cases but was not considered significant.

2. The average percentage increase in leucocytes was 89%.

3. As shown by differential counts the increase in cells was predominantly in the polymorphonuclear series.

4. There was also a lymphopenia with an average percentage drop in total lymphocyte counts of 55%.

5. The causative factor in the leucocytosis and the causative factor of the Jarisch-Herxheimer reaction are felt to be the same.

<sup>8</sup> Bierman, W., and Fishberg, E. H., *J. Am. Med. Assn.*, 1934, 103, 1354.

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# 17258. Occurrence of a Transient Leucocytosis During the Jarisch-Herxheimer Reaction.\*

FLETCHER McDOWELL. (Introduced by Bruce Webster.)

From the Department of Medicine, New York Hospital, and Cornell University Medical College.

There is no agreement in the medical literature on the characteristic blood picture in untreated early syphilis. Willcox<sup>1</sup> in a study of 405 patients with early syphilis found an average total white blood count of 8,950 with a normal differential. Previous to this report, findings of leucocytosis,<sup>2</sup> lymphocytosis,<sup>3</sup> monocytosis,<sup>4</sup> and eosinophilia<sup>5</sup> fill the literature on this subject.

No report exists, as far as can be determined, concerning the changes in the peripheral blood picture during a Jarisch-Herxheimer reaction. Sheldon and Heyman<sup>6,7</sup> have reported the occurrence of an acute polymorphonuclear leucocyte infiltration around early syphilitic lesions during a Jarisch-Herxheimer reaction, but they make no mention of the peripheral blood picture.

**Material.** Seventeen patients with early syphilis were used in this study. Of these 3 were seronegative primary, 6 were seropositive primary, and 8 were secondary. Four patients were female and 13 were male.

**Method.** After a diagnosis of syphilis was made and the pre-treatment white blood counts, differentials, and base line temperatures were completed, the patients were given a 300,000 unit dose of aqueous penicillin-G intramuscularly. They were then followed at

2 hourly intervals with total white blood counts, differentials and rectal temperatures. A total of 8 patients were followed with differential counts throughout the entire course of the reaction. All white counts were done by one observer, and 200 cells were counted in each differential. Periods of observation continued until the temperature returned to the pre-treatment level. The periods of observation varied from 10 to 16 hours in duration. At the end of the period of observation treatment was continued until a total of 2.4 million units of penicillin-G had been given over an 8-day period.

**Results.** Of the 17 patients, 15 had definite febrile Jarisch-Herxheimer reactions (defined here as in temperature to 37.5°C or above, occurring within 10 hours after the start of treatment and returning to normal within 16 to 18 hours). The distribution of the temperature elevations was as follows:

Temperature height	No. of patients.
37.8-38	3
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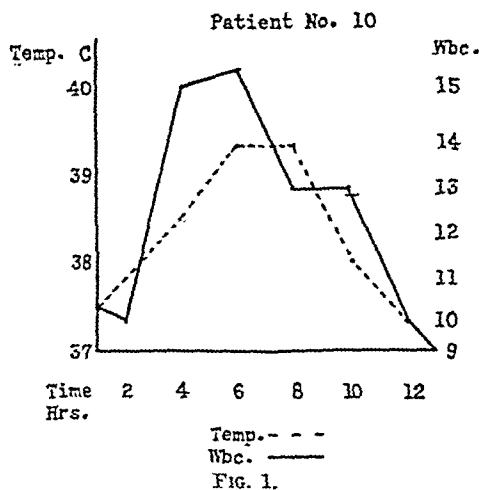
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<sup>6</sup> Sheldon and Heyman, *Recent Advances in the Study of Venereal Diseases*, 1948, p. 129.

<sup>7</sup> Sheldon, W. H., personal communication.

TABLE I.  
Deviations of Sodium and Potassium Determinations in Urine and Plasma by the Present Method of  
Flame Photometry from Sodium and Potassium Determinations by Chemical Methods.

Sodium					Potassium				
No.	Flame photometry, meq.	Gravimetric determin., meq.	Differ- ence, meq.	Differ- ence, %	No.	Flame photometry, meq.	Volumetric determin., meq.	Differ- ence, meq.	Differ- ence, %
1	147.1	143.9	+3.2	+2.2	1	3.83	3.74	+0.09	+2.4
2	144.9	143.7	+1.2	+0.8	2	3.40	3.29	+0.11	+3.3
3	143.8	144.7	-0.9	-0.6	3	4.30	4.40	-0.10	-2.3
4	145.8	143.7	+2.1	+1.5	4	4.76	4.67	+0.19	+1.9
5	112.0	111.8	+0.2	+0.2	5	4.48	4.38	+0.10	+2.3
6	146.8	144.8	+2.0	+1.4	6	4.70	4.64	+0.06	+1.3
7	135.2	137.8	-2.6	-1.9	7	4.46	4.48	-0.02	-0.4
8	140.5	143.3	-2.8	-2.0	8	4.42	4.41	+0.01	+0.2
9	142.5	140.0	+2.5	+1.8	9	5.59	5.61	-0.02	-0.4
10	133.7	135.5	-1.8	-1.3	10	4.65	4.82	-0.17	-3.5
11	138.8	140.0	-1.2	-0.8	11	4.85	4.73	+0.12	+2.5
12	135.5	136.2	-0.7	-0.5	12	4.68	4.54	+1.4	+3.1
13	91.0	88.7	+2.3	+2.6	Avg error 2.0% Range -3.5 to +3.3%				
14	92.3	91.0	+1.3	+1.4					
15	91.0	90.7	+0.3	+0.3					
16	180.8	178.1	+2.7	+1.5					
17	180.0	178.8	+1.2	+0.7	Avg error 1.2% Range -2.0 to +2.6%				
18	176.0	178.8	-2.8	-1.6					
19	142.0	143.1	-1.1	-0.8					
20	144.0	145.0	-1.0	-0.7					
21	139.0	138.3	+0.7	+0.5					

electrostatic effects due to variations of charge on the chamber walls.

The above conclusions were reached primarily from visual observations of the atomization of certain solutions containing fluorescein, under illumination with filtered ultraviolet light, under various standard laboratory conditions. For these reasons we decided to abandon the chamber and devise an atomizer which permitted the direct and immediate combustion of the atomized droplets.

Mention must also be made of the rather well-known spectroscopic phenomena of mutual excitation. This is illustrated by the production of more luminous energy per meq. of potassium in the presence of a large excess of sodium than from potassium in pure solution. This difficulty may be overcome by using as standards solutions containing a ratio of interfering elements, which only approximate that in the solution analyzed. For

instance, in the case of Na and K, there is a wide plateau of mutual excitation from 18 Na: 1K to 56 Na: 1K, wherein no increase of excitation occurs. A similar compensation is made for the less frequently observed phenomenon of quenching.

We have found it unnecessary to use an internal standard method with this atomizer-burner system. Direct experiment showed no interferences, such as reported by Barnes *et al.*,<sup>1,2</sup> from the presence in the solutions of the common inorganic acids up to 2% concentration, or of urea or sucrose up to at least 4% concentration.

Fig. 1 is a schematic illustration of the atomizer-burner system developed in order to achieve this result, and thus overcome the above mentioned difficulties. With this apparatus we have been able to introduce a constant amount of the atomized solution per unit time into a flame of constant thermal out-



## 17259. A New Method of Flame Photometry.\*

THEODORE E. WEICHELBAUM AND PHILIP L. VARNEY.

*From the Department of Surgery, and Department of Bacteriology and Immunology, Washington University School of Medicine, St. Louis, Mo.*

An adequate review of the principles and background of flame photometry is given in the papers of Barnes *et al.*<sup>1,2</sup>

In order to accomplish accurate quantitative analysis by flame photometry, the element to be determined must be in solution in the form of a salt and this solution must in some manner be atomized into a flame of constant thermal output, so that a constant amount of the solution is introduced into this flame per unit time.

All previous workers have utilized an atomizer-burner system which contained a chamber through which the atomized vapor of the solution passed before entering the exciting flame, and all commercial models of flame photometers which have been marketed in this country and abroad are based on this principle.

After working with such a chamber atomizer-burner system for many months, we found it impossible to obtain consistently reproducible and accurate quantitative results to within one per cent. Moreover, we could not consistently introduce a constant amount of solution into a flame per unit time. This was due mainly to the fact that when an aqueous solution is atomized and passed through a chamber, the size of the droplet particles which enter the exciting flame is not uniform. This factor is more or less uncontrollable, because the particle size and the rate of change of size are dependent upon numerous and variable physical conditions, among which are: (a) temperature of the atomizer chamber; (b) viscosity of the solution being atomized; (c) surface tension of the solution being atomized, and (d) certain

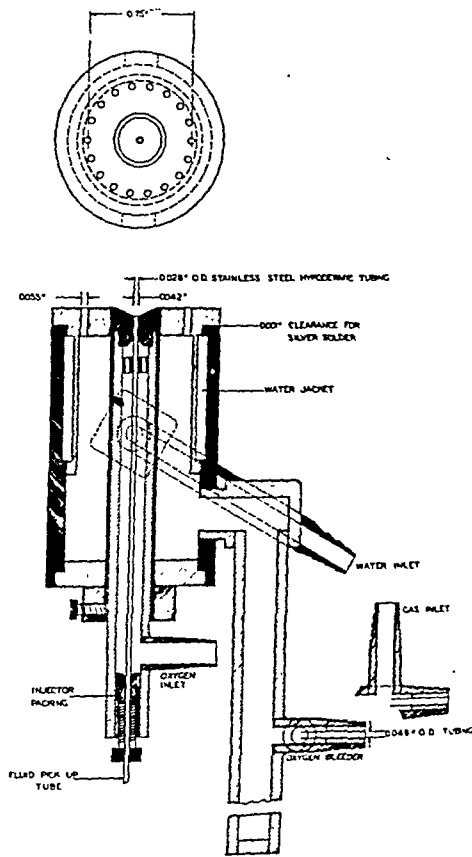


FIG. 1.

Diagram of Atomizer Burner System.

This system uses a mixture of illuminating or propane gas and oxygen for the thermal excitation of the atomized solutions. Atomization is accomplished by the use of oxygen under a constant pressure of 5 to 7 lbs., which enters the tube labeled oxygen-inlet and escapes at the narrow space below the fluid pick-up tube and the atomizer tip, producing a venturi effect, which accomplishes both the pick-up and atomization of the fluid to be analyzed. The fluid pick-up tube is immersed in a small glass beaker which contains the solution to be analyzed.

Approximately 0.75 ml of solution is atomized per minute, with a consumption of 11 cubic feet of oxygen per hour at 7 lbs. delivery pressure. To all solutions analyzed is added 0.04% STEROX SE (Monsanto Chemical Company), a non-ionic wetting agent, cation free, which overcomes the capillarity effects of the fluid pick-up tube.

\* This work was partially supported by a grant from the Commonwealth Fund of New York.

<sup>1</sup> Barnes, R. B., Richardson, D., Berry, J. W., and Hood, R. L., *Indust. and Eng. Chem., Anal. Ed.*, 1945, 17, 605.

<sup>2</sup> Berry, J. W., Chappell, D. G., and Barnes, R. G., *Indust. and Eng. Chem.*, 1946, 18, 19.

TABLE I.  
Effect of NaCN on Serum Alkaline Phosphatase Activity.

	Mols NaCN added to substrate					% initial activity remaining
	0 (Alkaline phosphatase, Bodansky units/100 cc serum)	0.001	0.005	0.01	0.05	
<i>Normal human subjects</i>						
1. Adult	3.0	2.1	0.8	0.5	—	16.7
2. "	2.7	1.6	0.6	0.3	0.4	11.1
3. "	2.1	1.8	0.8	0.6	0.5	23.8
4. "	2.0	1.5	0.5	0.3	0.4	20.0
5. Infants (pooled)	7.9	5.4	1.1	0.6	0.7	7.6
6. " "	6.0	—	1.0	0.9	1.0	16.7
<i>Obstructive jaundice</i>						
7. Biliary cirrhosis	46.7	28.8	11.9	3.2	—	6.9
8. Choledocholithiasis	27.2	—	7.4	2.0	1.4	5.1
9. Unclassified	22.5	—	4.7	1.4	1.3	5.8
10. ? Ca gall bladder	17.3	10.8	3.9	1.3	—	7.5
11. Cholangiolitic cirrhosis, marked parenchymal damage	16.2	—	2.7	1.2	0.9	5.6
12. Ca head of pancreas, cirrhosis	13.2	—	3.8	1.1	1.0	7.6
<i>Skeletal disorders</i>						
13. Paget's disease	75.9	—	11.3	3.3	2.4	3.2
14. Metastatic prostatic carcinoma	38.6	24.6	9.0	2.0	—	5.2
15. " " "	27.3	18.8	6.3	1.7	0.9	3.3
16. " " "	27.0	17.2	5.5	1.5	—	5.5
17. " " "	25.7	—	5.7	1.8	0.9	3.5
18. " " "	20.3	—	5.4	1.9	0.8	3.9
19. " " "	19.9	—	3.9	1.8	1.4	7.0
20. Paget's disease	9.1	—	2.4	0.6	0.6	6.6
21. Metastatic prostatic carcinoma	7.2	—	2.1	1.0	1.1	13.9

different from that normally present. We have repeated and extended these studies to include the effects of sodium cyanide on increased alkaline phosphatase levels in diseases of bone.

*Methods.* Serum alkaline phosphatase was determined by the Bodansky method.<sup>4</sup> A stock solution of 0.1M NaCN in buffered  $\beta$ -glycerophosphate solution was prepared and the pH of this solution adjusted to 9.2. Appropriate amounts of this stock solution were added to  $\beta$ -glycerophosphate substrate to make the desired cyanide concentration, the pH again checked, and the cyanide-substrate mixture then added to the serum just prior to incubation. Preliminary tests indicated that these concentrations of cyanide had no significant effect on the development of color by the inorganic phosphate standard.

*Results.* Four samples of normal adult human sera and 2 of pooled infants sera were examined and in each instance marked in-

hibition by cyanide was observed (Table I). The degree of inhibition increased with increasing concentrations of cyanide, at least up to 0.01M, although there was always some residual enzyme activity at the end of 1 hour incubation, varying from 0.4-1.0 Bodansky units % or 7.6-23.8% of the initial activity. The data indicate that most of the alkaline phosphatase present in normal human serum is inhibited by cyanide. These results are not in complete disagreement with those of Drill and Riggs<sup>3</sup> since examination of their data reveals inhibition greater than 50% in 2 of their 6 normal sera, and in several normal sera preincubation with NaCN disclosed inhibition of the order we obtained.

The data in 6 cases of obstructive jaundice with elevated serum alkaline phosphatase (Table I) confirm the previous finding<sup>3</sup> of marked inhibition by cyanide, to 5.1-7.6% of the initial activity in our series. In most instances we found the residual enzyme activity somewhat below the normal minimum of 1.5 Bodansky units %; it was higher only in

<sup>4</sup> Bodansky, A., *J. Biol. Chem.*, 1933, 101, 93.

put. Several different glass filter and interference filter photometric devices have been successfully utilized with this burner-atomizer system. These include a simple barrier layer photocell-suspension type galvanometer system, vacuum phototube-electronic voltmeter, and a direct current operated photomultiplier tube-suspension galvanometer system.

Comparison of the results of sodium and potassium determinations in urine and plasma by standard chemical methods,<sup>3,4</sup> with the method of flame photometry described herein, are shown in Table I. We do not believe that this comparison reflects the true accuracy of

this method of flame photometry, but represents at the present time the only acceptable means of evaluation.

The reproducibility, duplicability and recovery of added sodium and potassium to urine and plasma are of the order  $\pm 0.5\%$ . Further work is in progress for the determination of calcium, magnesium and other cations. Further data, as well as information as to the commercial availability of this instrument, will be available in a forthcoming publication.

*Summary.* A new type of atomizer-burner system is described for use in flame photometry which accomplishes consistently accurate quantitative chemical analysis of sodium, potassium, and other cations.

<sup>3</sup> Butler, A. M., and Tuthill, E., *J. Biol. Chem.*, 1931, **93**, 171.

<sup>4</sup> Folch, J., and Lauren, M., *J. Biol. Chem.*, 1947, **169**, 539.

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## 17260. Inhibition by Cyanide of Serum Alkaline Phosphatase in Normal Man, Obstructive Jaundice and Skeletal Disorders.

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*From the Department of Medicine, Columbia University, and the First (Columbia) Research Service, Goldwater Memorial Hospital, and the Presbyterian Hospital, New York City.*

It has been the general experience that marked increases in serum alkaline phosphatase activity occur consistently in only 2 disease categories in man: 1. in skeletal disorders associated with active, widespread proliferation of bone or cartilage, 2. in diseases of the liver or biliary tract with obstruction of the intra- or extrahepatic biliary channels.<sup>1</sup> There is general agreement that the high serum alkaline phosphatase levels observed in skeletal disorders are the result of augmented production of the enzyme by osteoblasts, which are known to be a prolific source. Opinion is divided as to the origin of the increased serum alkaline phosphatase in biliary tract obstruction. It would seem reasonable to suppose that since alkaline phosphatase is excreted in the bile and (being a large protein molecule) does not escape through intact glomeruli in man, retention of the enzyme in

the plasma occurs when the excretory biliary channels are obstructed. There is, however, the possibility that the increased enzyme appearing in the plasma is of hepatic origin, and perhaps a different alkaline phosphatase from that responsible for most of the enzyme activity exhibited by the plasma of normal subjects and of those with skeletal diseases.

Drill and coworkers<sup>2,3</sup> reported that sodium cyanide in concentrations to 0.1M only slightly inhibited the serum alkaline phosphatase activity of normal dogs and man whereas the elevated levels in hepatic damage were markedly reduced, to approximately normal levels but not below. Their data were interpreted as indicating that the alkaline phosphatase appearing in the serum of dogs and man with hepatic damage is, for the most part,

<sup>2</sup> Drill, V. A., Annegers, J. H., and Ivy, A. C., *J. Biol. Chem.*, 1944, **152**, 339.

<sup>3</sup> Drill, V. A., and Riggs, D. S., *J. Biol. Chem.*, 1946, **162**, 21.

<sup>1</sup> Gutman, A. B., Olson, K. B., Gutman, E. B., and Flood, C. A., *J. Clin. Invest.*, 1940, **19**, 129.

are maintained under these conditions and that the enzyme hence cannot be of hepatic origin.

Our data indicate that the increased serum alkaline phosphatase in obstructive jaundice, as in skeletal diseases, is phosphatase II (cyanide-sensitive) and therefore presumably not of hepatic cell origin. The results are compatible with the view that elevation of serum alkaline phosphatase in extra- or intrahepatic biliary tract obstruction is due to retention of serum alkaline phosphatase, largely of osseous origin. There is a great deal of clinical evidence for this view which was summarized elsewhere.<sup>1</sup>

The marked difference in the effects of cyanide on serum alkaline phosphatase and on liver tissue alkaline phosphatase indicates definite differences between these enzymes. However, the similarity between serum alkaline phosphatase and bone phosphatase with respect to cyanide does not prove their identity; all that can be said is that in this and other respects no significant difference is apparent. Absolute proof of identity of 2 enzymes from different sources is not possible at this time.

It may seem surprising that with so many alkaline phosphatases present in so many

organs, the enzyme in the plasma should be so preponderantly of osseous origin. However, not only is the number of osteoblasts in the body very large and their alkaline phosphatase production great, but the secretion of the enzyme is extracellular for production of bone at the cell surface. In most cells, phosphatases operate intracellularly, often apparently within the confines of the nucleus to judge from histochemical evidence, and probably never reach the extracellular fluids.

**Summary.** Cyanide markedly inhibits the serum alkaline phosphatase of normal human subjects and the increased levels of patients with obstructive jaundice and skeletal diseases; no essential differences were observed in these 3 categories. The evidence is consistent with the view that in all 3 categories the largest proportion of serum alkaline phosphatase is of osseous origin since bone phosphatase is inhibited by cyanide (Cloetens' phosphatase II) and liver phosphatases are, for the most part, cyanide-insensitive (Cloetens' phosphatase I). The data indicate that the rise in serum alkaline phosphatase in obstructive jaundice cannot be of hepatic origin but they are compatible with retention of serum alkaline phosphatase due to obstruction of the excretory biliary channels.

<sup>9</sup> Maddock, S., Schmidt, G., and Thannhauser, S. J., *Fed. Proc.*, 1942, 1, 181.

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From the McArdle Memorial Laboratory, Medical School, University of Wisconsin, Madison.

In studies<sup>1-4</sup> from this laboratory the intracellular distribution of riboflavin was de-

termined in normal rat livers, in the livers of rats fed various aminoazo dyes, and in liver tumors induced by 4-dimethylaminoazobenzene. Riboflavin is of particular interest since

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<sup>2</sup> Price, J. M., Miller, E. C., Miller, J. A., and Weber, G. M., *Cancer Research*, 1949, 9, in press.

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<sup>4</sup> Price, J. M., Miller, E. C., Miller, J. A., and Weber, G. M., *Cancer Research*, 1949, 9, 95.

1 serum with very marked initial activity.

In 9 patients with elevated serum alkaline phosphatase due to increased bone formation (in which the excess enzyme appearing in the serum is doubtless of osseous origin) the inhibitory effects of cyanide were indistinguishable from those observed in obstructive jaundice (Table I). The residual activity, 0.6-2.4 Bodansky units % was consistently less than the normal minimum except in the 2 cases with very high initial levels. The % activity persisting, 3.2-13.9% of the original values, was of the same order as observed in obstructive jaundice. A somewhat higher percentage of the original activity, approximately that observed in normal sera after inhibition by cyanide, persisted when initial levels were only moderately elevated.

Our data disclose no essential differences in the inhibiting effect of cyanide in all 3 categories; most of the serum alkaline phosphatase activity present normally, in obstructive jaundice and in skeletal disorders alike was inhibited by cyanide. Moreover, since 100% inhibition of an enzyme is infrequently achieved, the residual enzyme activity, low as it was in most sera, probably represents maximal values for cyanide-insensitive alkaline phosphatases present in serum. That we apparently did not obtain complete inhibition of cyanide-sensitive phosphatases is indicated by the persistence of greater residual activity in sera with higher initial levels.

**Discussion.** The data obtained appear to throw light upon 2 obscure points, 1. the origin of the alkaline phosphatases in the plasma of normal human adults\*, 2. the origin and causal mechanism of the increased serum alkaline phosphatase in obstruction of the extra- or intrahepatic biliary tract.

Cloetens<sup>5</sup> distinguished 2 classes of alkaline phosphatase: Phosphatase I, inactive in the absence of  $Mg^{++}$ , markedly activated by appropriate concentrations of  $Mg^{++}$ , not inhibited by cyanide when so activated; phos-

phatase II, active without addition of  $Mg^{++}$  and only little affected by addition of  $Mg^{++}$  but markedly inhibited by cyanide. Cloetens<sup>5</sup> found that phosphatase II, cyanide-sensitive, accounted for over 90% of the total alkaline phosphatase activity of bone (which we have confirmed in unpublished experiments; see also O. Bodansky<sup>6</sup>), 96-99% of that of the serum of 3 rachitic dogs, and 97% of that of the serum of 1 dog with liver disease. Liver tissue phosphatase, in contrast, was usually more than 60% phosphatase I, cyanide-resistant.<sup>7</sup> In our experiments,<sup>†</sup> inhibition by cyanide of 76.2-92.4% of the alkaline phosphatase activity of normal human sera indicates that most of this activity is due to a phosphatase II, like bone phosphatase but unlike most liver phosphatases. Belfanti *et al.*,<sup>7</sup> using oxalate inhibition as a criterion, also found that the alkaline phosphatase of normal serum resembled bone phosphatase but differed from liver phosphatase. The largest proportion of the alkaline phosphatases present in normal human serum would therefore appear from this chemical evidence to be indistinguishable by available methods from bone phosphatase; a small proportion, cyanide-resistant, is probably not of osseous but of other (undetermined) origin. This interpretation is consistent with observations in hepatectomized and eviscerated dogs<sup>8,9</sup> indicating that serum alkaline phosphatase levels

<sup>5</sup> Cloetens, R., *Enzymologia*, 1939, 6, 46.

<sup>6</sup> Bodansky, O., *J. Biol. Chem.*, 1949, 179, 81.

<sup>†</sup> Presumably, much of the cyanide-sensitive phosphatase II found in liver is serum phosphatase in the blood and bile present in liver tissue preparations.

<sup>‡</sup> We did not examine the effects of addition of magnesium ions (see Drill and Riggs<sup>10</sup>) because the presence of magnesium in serum makes interpretation difficult. Moreover, Cloetens worked largely with dialysed tissue extracts and the differences he noted in the effects of addition of  $Mg^{++}$  probably reflect differences in the ease with which  $Mg$  ion is removed by dialysis from the various alkaline phosphatases of different organs. This factor does not enter into our experiments.

<sup>7</sup> Belfanti, S., Contardi, A., and Ercoli, A., *Biochem. J.*, 1935, 29, 1491.

<sup>8</sup> Armstrong, A. R., and Banting, F. G., *Can. Med. Assn. J.*, 1935, 33, 243.

\* It is generally accepted that the increased serum alkaline phosphatase of growing children, like that of patients with bone or cartilage proliferation due to skeletal disorders, is due to increased osteoblastic activity.

are maintained under these conditions and that the enzyme hence cannot be of hepatic origin.

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high dietary levels of this vitamin delay the induction of neoplasms by certain of the aminoazo dyes.<sup>5</sup> Furthermore, ingestion of any one of the various dyes lowers the level of riboflavin in the liver to a degree that is roughly proportional to its carcinogenic activity.<sup>6,7</sup> While the ingestion of 4-dimethylaminoazobenzene also decreases the level of vitamin B<sub>6</sub> in the liver this vitamin differs from riboflavin in that the various dietary changes which enhance or lower the carcinogenic activity of this dye do not alter the level of hepatic vitamin B<sub>6</sub> in a significant fashion.<sup>7</sup> Hence it was desirable to compare the intracellular distribution of vitamin B<sub>6</sub> with that of riboflavin in the livers of rats before and after the administration of 4-dimethylaminoazobenzene as well as in hepatic tumors induced by this dye. Similar data for the liver of the mouse, a species relatively resistant to the carcinogenic action of this dye,<sup>5,8</sup> are also presented.

**Methods.** The animals were fed a semi-synthetic diet (diet 3<sup>7</sup>) containing 1.2 mg of riboflavin per kg. Male albino rats<sup>†</sup> were fed this basal diet with or without 0.06% 4-dimethylaminoazobenzene for 4 weeks. Female albino mice<sup>‡</sup> were fed the same diets but for 4 months. The animals were killed with ether and the livers perfused *in situ* with isotonic saline. The perfusion and all subsequent steps prior to the analytical procedures were carried out at 0 to 5°. The rat liver tumors were obtained from rats fed the basal diet plus the dye for 4 to 5 months. Nine mouse livers were pooled for fractionation No. 1 and 7 livers were combined for fractionation no. 2. Each fractionation of rat liver was made on pools from 2 (fraction-

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Differential centrifugation was used as described previously<sup>1,2,4</sup> to prepare the nuclear, large granule (mitochondria), small granule (microsome), and supernatant fluid fractions. The nuclear and large granule fractions were washed twice; the small granules were sedimented at 24,000 × g (at center of tube) for 3 hours and were not washed. In experiments where the small granules were washed the nuclear and large granule fractions were sedimented in the usual manner, but were washed only once. The supernatant fluid and washing from the large granules were combined and centrifuged for 36 minutes at 120,000 × g (at center of tube) in an air-driven ultracentrifuge. The small granules were then resuspended in the sucrose solution and, after aliquots had been taken for analysis, were brought to the volume in which they had previously been suspended. After recentrifugation under identical conditions the sediment was suspended in the sucrose solution for analysis.

Vitamin B<sub>6</sub> was determined with *Saccharomyces carlsbergensis* by the method of Atkin *et al.*<sup>9</sup> This yeast responds equally to the 3 known forms of vitamin B<sub>6</sub>.<sup>10</sup> The intracellular distributions of protein, nucleic acids, and riboflavin were also determined as previously described.<sup>1</sup> The intracellular distributions of these constituents in the fractionations of the rat tissues agreed well with those previously published.<sup>1,4</sup>

**Results.** From Table I it is evident that most of the vitamin B<sub>6</sub> was in the large granules and the supernatant fluid in all of the tissues studied. The large granules con-

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TABLE I.  
 Intracellular Distribution of Vitamin B<sub>6</sub> in Liver and Liver Tumor.

Fraction	Mouse livers		Rat livers		Rat liver tumors		
	Basal diet	Basal diet + dye	Basal diet	Basal diet + dye			
	1	2	3	4	5	6	8
	Fractionation No.						
	$\mu\text{g}$ of vitamin B <sub>6</sub> per g of fresh tissue						
Whole homogenate	6.0	4.5	6.4	6.4	4.0	4.7	1.8
Nuclei	0.5	0.5	0.4	0.2	0.3	0.2	0.2
Large granules	2.0	1.4	2.3	2.9	1.6	1.9	0.5
Small "	0.3	0.2	0.3	0.4	0.2	0.2	0.2
Supernatant fluid	3.0	2.2	3.0	3.1	1.8	2.1	1.2
Recovery	5.8	4.3	6.0	6.6	3.9	4.4	2.1
	$\mu\text{g}$ of vitamin B <sub>6</sub> per g of protein						
Whole homogenate	46	34	53	50	37	40	15
Nuclei	22	18	26	14	18	11	5
Large granules	57	57	60	73	60	58	46
Small "	19	16	18	21	17	13	12
Supernatant fluid	60	36	64	62	40	41	26

tained 28 to 45% and the supernatant fluid 45 to 64% of the total amount of vitamin B<sub>6</sub>. The ratio of vitamin B<sub>6</sub> to protein was higher in the large granule and supernatant fluid fractions, and lower in the nuclear and small granule fractions than it was in the whole homogenate. When 4-dimethylaminoazobenzene was included in the diets of either rats (fractionations no. 5 and 6) or mice (fractionation no. 2) the amount of vitamin B<sub>6</sub> in the large granule and supernatant fluid fractions was considerably reduced. Since the protein content was also reduced in the large granule fraction<sup>1,2</sup> the ratio of vitamin to protein was unchanged in this fraction. On the other hand this ratio was decreased in the supernatant fluid since the protein content of this fraction remained unchanged.<sup>1,2</sup> In the liver tumor tissue the level of vitamin B<sub>6</sub> in the large granules and supernatant fluid was even lower than in the livers of rats fed the dye, and the ratio of the vitamin to protein was also much lower.

The small granules isolated from the livers of rats fed the basal diet after centrifuging at 120,000  $\times$  g for 36 minutes were entirely comparable in their contents of pentosenucleic acid, riboflavin and vitamin B<sub>6</sub> to those isolated from similar livers at 24,000  $\times$  g for 3 hours. A single washing reduced the level of vitamin B<sub>6</sub> in these particles by 50 to 80%.

The pentosenucleic acid was reduced by 13 to 28% and in a single experiment no detectable loss of riboflavin occurred on washing. These changes are probably attributable in part to the removal of some residual supernatant fluid from the small granules.

*Discussion.* As in the case of riboflavin<sup>1-4</sup> the vitamin B<sub>6</sub> was found chiefly in the large granules and supernatant fluid in all the tissues studied. The nuclear fraction of normal rat liver contained 3 to 6% of the total vitamin B<sub>6</sub> whereas this fraction generally contains 5 to 9% of the total riboflavin. The greatest differences in the distribution of the two vitamins was found in the small granules. The washed small granules contained only 0.8 to 1.9% of the total vitamin B<sub>6</sub> but still contained 16% of the total riboflavin. It is probable that the values for the vitamin B<sub>6</sub> and riboflavin contents of the nuclear fraction are too high, since this fraction is contaminated with some whole cells and large granules.<sup>1,11</sup>

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high dietary levels of this vitamin delay the induction of neoplasms by certain of the aminoazo dyes.<sup>5</sup> Furthermore, ingestion of any one of the various dyes lowers the level of riboflavin in the liver to a degree that is roughly proportional to its carcinogenic activity.<sup>6,7</sup> While the ingestion of 4-dimethylaminoazobenzene also decreases the level of vitamin B<sub>6</sub> in the liver this vitamin differs from riboflavin in that the various dietary changes which enhance or lower the carcinogenic activity of this dye do not alter the level of hepatic vitamin B<sub>6</sub> in a significant fashion.<sup>7</sup> Hence it was desirable to compare the intracellular distribution of vitamin B<sub>6</sub> with that of riboflavin in the livers of rats before and after the administration of 4-dimethylaminoazobenzene as well as in hepatic tumors induced by this dye. Similar data for the liver of the mouse, a species relatively resistant to the carcinogenic action of this dye,<sup>5,8</sup> are also presented.

**Methods.** The animals were fed a semi-synthetic diet (diet 3<sup>7</sup>) containing 1.2 mg of riboflavin per kg. Male albino rats<sup>†</sup> were fed this basal diet with or without 0.06% 4-dimethylaminoazobenzene for 4 weeks. Female albino mice<sup>‡</sup> were fed the same diets but for 4 months. The animals were killed with ether and the livers perfused *in situ* with isotonic saline. The perfusion and all subsequent steps prior to the analytical procedures were carried out at 0 to 5°. The rat liver tumors were obtained from rats fed the basal diet plus the dye for 4 to 5 months. Nine mouse livers were pooled for fractionation No. 1 and 7 livers were combined for fractionation no. 2. Each fractionation of rat liver was made on pools from 2 (fraction-

ations no. 3 and 5) or 3 (fractionations no. 4 and 6) animals. Small tumors which were grossly non-necrotic were pooled from 9 and 20 rats for fractionations no. 7 and 8, respectively. The selection of the tumors, which were obtained from non-perfused livers, was made as previously described.<sup>4</sup> The pooled tumors and livers were forced through a plastic tissue mincer and homogenized in 0.88 M sucrose solution as previously described.<sup>1</sup>

Differential centrifugation was used as described previously<sup>1,2,4</sup> to prepare the nuclear, large granule (mitochondria), small granule (microsome), and supernatant fluid fractions. The nuclear and large granule fractions were washed twice; the small granules were sedimented at 24,000 x g (at center of tube) for 3 hours and were not washed. In experiments where the small granules were washed the nuclear and large granule fractions were sedimented in the usual manner, but were washed only once. The supernatant fluid and washing from the large granules were combined and centrifuged for 36 minutes at 120,000 x g (at center of tube) in an air-driven ultracentrifuge. The small granules were then resuspended in the sucrose solution and, after aliquots had been taken for analysis, were brought to the volume in which they had previously been suspended. After recentrifugation under identical conditions the sediment was suspended in the sucrose solution for analysis.

Vitamin B<sub>6</sub> was determined with *Saccharomyces carlsbergensis* by the method of Atkin *et al.*<sup>9</sup> This yeast responds equally to the 3 known forms of vitamin B<sub>6</sub>.<sup>10</sup> The intracellular distributions of protein, nucleic acids, and riboflavin were also determined as previously described.<sup>1</sup> The intracellular distributions of these constituents in the fractionations of the rat tissues agreed well with those previously published.<sup>1,4</sup>

**Results.** From Table I it is evident that most of the vitamin B<sub>6</sub> was in the large granules and the supernatant fluid in all of the tissues studied. The large granules con-

<sup>5</sup> Rusch, H. P., Baumann, C. A., Miller, J. A., and Kline, B. E., in Moulton, F. R., A.A.A.S. research conference on cancer, Washington, 1945, 267.

<sup>6</sup> Griffin, A. C., and Baumann, C. A., *Arch. Biochem.*, 1946, 11, 467.

<sup>7</sup> Miller, E. C., Miller, J. A., Kline, B. E., and Rusch, H. P., *J. Exp. Med.*, 1948, 88, 89.

<sup>8</sup> Kirby, A. H. M., *Cancer Research*, 1945, 5, 683.

<sup>†</sup> Holtzman Rat Company, Madison, Wis.

<sup>‡</sup> A. Sutter, Springfield, Mo.

<sup>9</sup> Atkin, L., Schultz, A. S., Williams, W. L., and Frey, C. N., *Ind. and Eng. Chem., Anal. Ed.*, 1943, 15, 141.

<sup>10</sup> Snell, E. E., *Physiol. Rev.*, 1948, 28, 255.

TABLE I.  
 Intracellular Distribution of Vitamin B<sub>6</sub> in Liver and Liver Tumor.

Fraction	Mouse livers		Rat livers			Rat liver tumors	
	Basal diet	Basal diet + dye	Basal diet	Basal diet + dye			
	1	2	3	4	5	6	7
							8
			Fractionation No.				
			$\mu\text{g}$ of vitamin B <sub>6</sub> per g of fresh tissue				
Whole homogenate	6.0	4.5	6.4	6.4	4.0	4.7	1.8
Nuclei	0.5	0.5	0.4	0.2	0.3	0.2	0.2
Large granules	2.0	1.4	2.3	2.9	1.6	1.9	0.5
Small "	0.3	0.2	0.3	0.4	0.2	0.2	0.2
Supernatant fluid	3.0	2.2	3.0	3.1	1.8	2.1	1.2
Recovery	5.8	4.3	6.0	6.6	3.9	4.4	2.1
							1.3
			$\mu\text{g}$ of vitamin B <sub>6</sub> per g of protein				
Whole homogenate	46	34	53	50	37	40	15
Nuclei	22	18	26	14	18	11	5
Large granules	57	57	60	73	60	58	46
Small "	19	16	18	21	17	13	12
Supernatant fluid	60	36	64	62	40	41	26
							14

tained 28 to 45% and the supernatant fluid 45 to 64% of the total amount of vitamin B<sub>6</sub>. The ratio of vitamin B<sub>6</sub> to protein was higher in the large granule and supernatant fluid fractions, and lower in the nuclear and small granule fractions than it was in the whole homogenate. When 4-dimethylaminoazobenzene was included in the diets of either rats (fractionations no. 5 and 6) or mice (fractionation no. 2) the amount of vitamin B<sub>6</sub> in the large granule and supernatant fluid fractions was considerably reduced. Since the protein content was also reduced in the large granule fraction<sup>1,2</sup> the ratio of vitamin to protein was unchanged in this fraction. On the other hand this ratio was decreased in the supernatant fluid since the protein content of this fraction remained unchanged.<sup>1,2</sup> In the liver tumor tissue the level of vitamin B<sub>6</sub> in the large granules and supernatant fluid was even lower than in the livers of rats fed the dye, and the ratio of the vitamin to protein was also much lower.

The small granules isolated from the livers of rats fed the basal diet after centrifuging at 120,000  $\times$  g for 36 minutes were entirely comparable in their contents of pentosenucleic acid, riboflavin and vitamin B<sub>6</sub> to those isolated from similar livers at 24,000  $\times$  g for 3 hours. A single washing reduced the level of vitamin B<sub>6</sub> in these particles by 50 to 80%.

The pentosenucleic acid was reduced by 13 to 28% and in a single experiment no detectable loss of riboflavin occurred on washing. These changes are probably attributable in part to the removal of some residual supernatant fluid from the small granules.

*Discussion.* As in the case of riboflavin<sup>1-4</sup> the vitamin B<sub>6</sub> was found chiefly in the large granules and supernatant fluid in all the tissues studied. The nuclear fraction of normal rat liver contained 3 to 6% of the total vitamin B<sub>6</sub> whereas this fraction generally contains 5 to 9% of the total riboflavin. The greatest differences in the distribution of the two vitamins was found in the small granules. The washed small granules contained only 0.8 to 1.9% of the total vitamin B<sub>6</sub> but still contained 16% of the total riboflavin. It is probable that the values for the vitamin B<sub>6</sub> and riboflavin contents of the nuclear fraction are too high, since this fraction is contaminated with some whole cells and large granules.<sup>1,11</sup>

Claude<sup>12</sup> stated that he found most of the transaminase activity of rat and guinea pig liver in the supernatant fluid. The trans-

<sup>11</sup> Hogeboom, G. H., Schneider, W. C., and Palade, G. E., *J. Biol. Chem.*, 1948, **172**, 619.

<sup>12</sup> Claude, A., in Moulton, F. R., A.A.A.S. research conference on cancer, Washington, 1945, 223.

minase activity was relatively low in the large granules and was completely absent from the microsome or small granule fraction. Subsequent to these observations it was found that vitamin B<sub>6</sub> is an integral part of this enzyme system.<sup>13</sup> Thus the data presented above support those of Claude since the small granules contain no transaminase activity and very little vitamin B<sub>6</sub> while the parts of the liver cell that contain transaminase activity are particularly rich in vitamin B<sub>6</sub>. No information is available on the presence or distribution in these tissues of other enzymes which contain vitamin B<sub>6</sub>.

The hepatic carcinogen 4-dimethylaminoazobenzene reduced the level of riboflavin<sup>1,2</sup> and vitamin B<sub>6</sub> in the large granules and supernatant fluid, but in both cases the reduction of protein in the large granules paralleled the decrease in vitamin content. This is probably the result of a reduction of the number of large granules in the liver cells.<sup>3</sup>

**Summary.** The intracellular distribution of vitamin B<sub>6</sub> in rat liver tumors induced by 4-dimethylaminoazobenzene and in the livers

of rats and mice before and after administration of the azo dye was determined after differential centrifugation of the tissue homogenates in hypertonic sucrose solution.

In all cases vitamin B<sub>6</sub> was concentrated in the washed large granules or mitochondria (28 to 45% of total) and in the supernatant fluids (45 to 64% of total). The washed nuclear fractions contained 3 to 11% of the total vitamin B<sub>6</sub> present. The unwashed small granules or microsomes contained 4 to 8% of the total vitamin B<sub>6</sub>; in the case of normal rat liver one washing reduced the level to about 1½%.

The ingestion of the azo dye reduced the levels of the vitamin in the large granule and supernatant fluid fractions of the livers in each species. The decrease in protein content of the large granules paralleled the decrease in vitamin B<sub>6</sub> content. The large granule and supernatant fluid fractions of the liver tumors contained even less vitamin B<sub>6</sub> than was found in the corresponding fractions from the livers of rats fed the dye and the ratio of the vitamin to protein was also much lower.

<sup>13</sup> Wynne. A. M., in *Ann. Rev. of Biochem.*, 1946, 15, 58.

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## 17262. Healing of Tuberculous Pulmonary Cavities by Means of Skin Grafts.

ARTHUR MARTIN VINEBERG. (Introduced by J. S. L. Browne.)

*From Grace Dart Home Hospital, Montreal, Canada.*

Giant tuberculous pulmonary cavities continue to present one of the most complex problems in the surgical treatment of pulmonary tuberculosis. Various types of collapse therapy have resulted in a high percentage of failures. Intracavitary drainage in tension or blocked cavities, combined with collapse therapy, has greatly increased the number of good results.

Intracavitary drainage without collapse therapy provides relief from toxicity but is of little value in cavity closure. In advanced pulmonary disease with multiple cavitation, collapse therapy is contraindicated.

We wish to present a somewhat different point of view. Let us consider that a tuberculous pulmonary cavity is a chronic lung abscess: The lung surrounding a chronic non-specific lung abscess shows little disease. When such an abscess is drained, the surrounding lung tissues fill in the defect occupied by the abscess. In a tuberculous lung abscess, the surrounding lung tissues are usually diseased and cannot expand to fill in the space occupied by the abscess cavity. Drainage is of cleansing value in pulmonary tuberculous cavities, as in other types of pulmonary abscesses, but cavity obliteration does not occur without col-



FIG. 1.

Five months following cavernostomy and repeated skin grafts the lipiodol is lying on the skin lining the former cavity which is continuous with the skin of the chest wall and does not enter the bronchial tree.

lapse therapy.

At the Grace Dart Home Hospital large cavities have been treated by intracavitary drainage. The residual cavity space has been opened into by cavernostomy, carried out through the sinus tract of the drainage tube. The exposed cavity walls and floor have been covered with split-thickness skin grafts or pinch grafts. Gradually the boundaries of the cavity are lined with skin which grows out to meet the skin on the surface of the chest wall. The cavity thus becomes obliterated, leaving a defect in the chest wall.

The first case selected was a 52-year-old male with far-advanced bilateral pulmonary

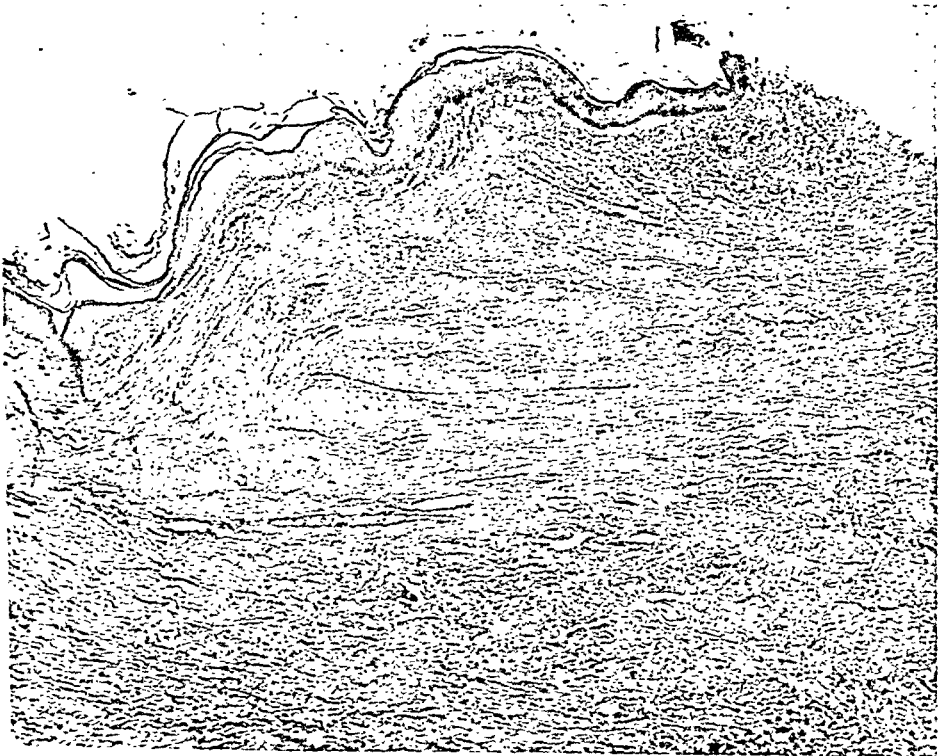


FIG. 2.

Section taken through wall of skin grafted cavity. Note lung covered by a layer of skin with proliferation of fibrous tissue and compressed pulmonary alveoli.

minase activity was relatively low in the large granules and was completely absent from the microsome or small granule fraction. Subsequent to these observations it was found that vitamin B<sub>6</sub> is an integral part of this enzyme system.<sup>13</sup> Thus the data presented above support those of Claude since the small granules contain no transaminase activity and very little vitamin B<sub>6</sub> while the parts of the liver cell that contain transaminase activity are particularly rich in vitamin B<sub>6</sub>. No information is available on the presence or distribution in these tissues of other enzymes which contain vitamin B<sub>6</sub>.

The hepatic carcinogen 4-dimethylaminoazobenzene reduced the level of riboflavin<sup>1,2</sup> and vitamin B<sub>6</sub> in the large granules and supernatant fluid, but in both cases the reduction of protein in the large granules paralleled the decrease in vitamin content. This is probably the result of a reduction of the number of large granules in the liver cells.<sup>3</sup>

*Summary.* The intracellular distribution of vitamin B<sub>6</sub> in rat liver tumors induced by 4-dimethylaminoazobenzene and in the livers

of rats and mice before and after administration of the azo dye was determined after differential centrifugation of the tissue homogenates in hypertonic sucrose solution.

In all cases vitamin B<sub>6</sub> was concentrated in the washed large granules or mitochondria (28 to 45% of total) and in the supernatant fluids (45 to 64% of total). The washed nuclear fractions contained 3 to 11% of the total vitamin B<sub>6</sub> present. The unwashed small granules or microsomes contained 4 to 8% of the total vitamin B<sub>6</sub>; in the case of normal rat liver one washing reduced the level to about 1½%.

The ingestion of the azo dye reduced the levels of the vitamin in the large granule and supernatant fluid fractions of the livers in each species. The decrease in protein content of the large granules paralleled the decrease in vitamin B<sub>6</sub> content. The large granule and supernatant fluid fractions of the liver tumors contained even less vitamin B<sub>6</sub> than was found in the corresponding fractions from the livers of rats fed the dye and the ratio of the vitamin to protein was also much lower.

<sup>13</sup> Wynne, A. M., in *Ann. Rev. of Biochem.*, 1946, 15, 58.

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We wish to present a somewhat different point of view. Let us consider that a tuberculous pulmonary cavity is a chronic lung abscess: The lung surrounding a chronic non-specific lung abscess shows little disease. When such an abscess is drained, the surrounding lung tissues fill in the defect occupied by the abscess. In a tuberculous lung abscess, the surrounding lung tissues are usually diseased and cannot expand to fill in the space occupied by the abscess cavity. Drainage is of cleansing value in pulmonary tuberculous cavities, as in other types of pulmonary abscesses, but cavity obliteration does not occur without col-

aorta with rubber-tipped bulldog clamps. Each animal was then given 150 mg/kg of 5% alloxan (Eastman Kodak) into an ear vein during a 2 minute period and the clamps were left in place for a further period of 8 minutes. The clamps were then removed and the incision closed. Blood samples were obtained immediately before the alloxan injection and at frequent intervals thereafter, and the blood sugar was determined by the Folin and Malmros micromethod.<sup>3</sup>

The animals were sacrificed by an intravenous injection of sodium pentobarbital. A part of the pancreas was removed immediately and fixed in Bouin's fluid. The remainder of the pancreas and portions of other organs were fixed in Helly's fluid. Gomori's chrome alum hematoxylin stain<sup>4</sup> was used to differentiate beta and alpha cells in the islets of Langerhans, and hematoxylin and eosin was used on the other tissues.

*Preliminary Experiments.* In some preliminary experiments the celiac artery alone was clamped. Of 8 such animals given alloxan 2 developed severe diabetes at 24 hours with typical destruction of the pancreatic islets. Two others developed mild, transient, delayed hyperglycemia after 6 days but when studied histologically at 19 and 21 days no abnormalities were detected in the pancreas. The remaining 4 failed to show hyperglycemia in 19 to 37 days of study and on histological examination the pancreas appeared normal. Of the 6 animals which failed to develop typical diabetes 5 had shown definite early hypoglycemia with convulsions in 3 cases.

When India ink (20 cc of a 1 to 5 dilution) was injected into an ear vein with only the celiac artery clamped small quantities of the ink were detected in the pancreas indicating that the blood supply was not completely occluded. However, when both the celiac and mesenteric arteries were clamped and India ink injected no ink could be detected with certainty in the pancreas of 2 animals while in a 3rd only very small quantities were present. Control sections of the liver and kidney

TABLE I.  
Effect of Alloxan on Blood Sugar of Rabbits with Celiac and Superior Mesenteric Arteries Occluded.

Rabbit No.	Control†	Min.		Blood sugar in mg/100 cc																Notes
				Hr								Days								
		20	40	1	2	3	4	5	6	7	8	9	1	2	3	4	5	6	7	
1	127	177	152	142	133	87	124	106	97			70	137		93	104		99	105	Sacrificed 15 days
2	114	142	122	122	118	92	94	84	92			124	116		135	121		127	118	" 15 "
3	103	87	87	103	84	66	47*	65	65			96	108	114						" 3 "
4	114	127	137	153	148	139	87	70	57	43*		124	116	122						" 3 "
5	84	92	89	148	171	131	81	41*	50			159	—	139	171	258	230			" 6 "
6	94	93	84	87	75	45	34*					59	118							Died
7	101	109	114	109	150	101	88	73	37*			79	129	85				116	109	Sacrificed 12 "
8	99	120	128	131	114	112	93	82	69			96	105	92				108	116	" 12 "
9	89	81	85	90	120	127	105	106		62	52	46	106	116	112					" 5 "
10	103	81	101	112	135	118	97	68	59	60		175	156	153						" 5 "

\* Hypoglycemic reaction.

† After induction of anesthesia, immediately before alloxan injection.

<sup>3</sup> Folin, O., and Malmros, H. J., *J. Biol. Chem.*, 1929, 83, 115.

<sup>4</sup> Gomori, G., *Am. J. Path.*, 1941, 17, 395.



disease with giant bilateral apical cavities. On February 4, 1948, an anterior stage thoracoplasty was done on the left side. On May 28, 1948, intracavitary drainage was established in the left apical cavity. The poor general condition of the patient prevented further collapse therapy. On September 22, 1948, the left apical cavity was entered into through the drainage tract. Cavernostomy was done, and the depths of the cavity were lined with a split-thickness skin graft. This was repeated on October 20, 1948, November 17, 1948 and on February 9, 1949; on the last occasion pinch grafts were used. In all instances mentioned, at least a 75% "take" of the graft was obtained. The cavity on the left side

diminished and gradually was covered with skin; the bronchial openings, which at first were large, gradually closed. At autopsy, May 8, 1949, there was no evidence of an open bronchus leading to the skin surface. The surface skin on the chest wall showed a depression about  $1\frac{1}{2}$ " in diameter which entered into the lung. The cavity seemed to be completely covered by skin and was entirely exteriorized. The cavity on the right side remained unchanged. Two other cases have been treated. In both skin grafting has been successful and the cavities seem to be healing.

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### 17263. Effect of Alloxan in Rabbits with Temporary Occlusion of the Arteries to the Pancreas.\*

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*From the George F. Baker Clinic, New England Deaconess Hospital, Boston, Mass.*

Numerous investigations have shown that the injection of alloxan into various animals causes destruction of the pancreatic islets of Langerhans with the production of diabetes. After the injection of a diabetogenic dose of alloxan intravenously into a rabbit hyperglycemia appears which reaches its peak within 2 hours. This in turn is followed in 2 to 9 hours by profound hypoglycemia frequently with convulsions. Finally within 24 to 48 hours diabetes develops and although most agree that the diabetes results from the destruction of the islets of Langerhans, the initial hyperglycemic phase and especially the hypoglycemic phase have caused much speculation and disagreement.

Although diabetes usually is considered to result from an action of the drug on the pancreatic islets, Jimenez-Diaz<sup>1</sup> reported that clamping the renal blood vessels immediately

before the injection of alloxan prevents the development of diabetes. Others<sup>2</sup> have not been able to confirm this.

In the present investigation alloxan was injected into rabbits with the blood supply to the pancreas occluded temporarily in order (1) to confirm that alloxan diabetes is pancreatic in origin, and (2) to determine if the hypoglycemia is dependent upon alloxan producing histological changes in the islets of Langerhans.

*Materials and Methods.* Male albino rabbits weighing 2.0 to 2.7 kg were used and each was fasted for 16 to 18 hours before an experiment. Anesthesia was obtained by 18 to 20 mg/kg of 5% sodium pentobarbital intravenously supplemented by ether given by open mask as required. A transverse incision was made in the left upper quadrant of the abdomen extending quite far laterally. The celiac axis and the superior mesenteric artery were exposed and clamped close to the abdominal

\* This work was aided by grants from the American Cyanamid Co., and the Diabetic Fund.

<sup>1</sup> Jimenez-Diaz, G., Grande-Covian, F., and DeOya, J. C., *Nature*, 1946, **158**, 589.

<sup>2</sup> Martinez, C., Gitter, S., and Covian, M. R., *Rev. Soc. argent de biol.*, 1947, **223**, 81.

glycemic phase is either extrapancreatic in origin as first suggested by Houssay<sup>5</sup> or that insulin is released from the islets by the action of a small quantity of alloxan insufficient to produce detectable histological changes.

After these studies had been completed we learned that Carrasco-Formiguera<sup>6</sup> had conducted experiments in 4 dogs in which alloxan, in dosage of 75 mg/kg, was injected into an intramesenteric vein "... while the pancreas was totally excluded from the circulation by clamping all its visible channels of irrigation, previous to, through 6 minutes after the said injection ...". All 4 dogs developed profound hypoglycemia and in the 2 animals whose tissues were examined histologically the pancreas was said to be normal but the liver showed extremely severe lesions. Carrasco-Formiguera concluded from these experiments that the probable cause of allox-

an hypoglycemia was liver damage. However, in our rabbits with occluded pancreatic circulation the alloxan was injected into an ear vein instead of into the portal system and hypoglycemia developed without detectable histological changes in the liver.

**Summary.** Diabetes was prevented in 9 of 10 rabbits given alloxan by temporary occlusion of the celiac and superior mesenteric arteries. The remaining animal developed moderate, delayed diabetes with mild histological changes in the islets of Langerhans.

Severe hypoglycemia was observed in several of the rabbits which failed to develop diabetes and which failed to show any histological change in the islets of Langerhans or the liver when examined at 2 to 15 days.

These experiments indicate that alloxan diabetes is pancreatic in origin but suggest that the hypoglycemic phase is either extrapancreatic in origin or may be produced by the action of very small quantities of alloxan insufficient to produce detectable changes in the islets of Langerhans.

Received July 1, 1949. P.S.E.B.M., 1949, 71.

<sup>5</sup> Houssay, B. A., Orias, O., and Sara, I., *Science*, 1945, 102, 197.

<sup>6</sup> Carrasco-Formiguera, R., *Arch. Biol. Pat. (Univ. de Los Andes)*, 1948, 1, 111.

## 17264. Effect of Uric Acid in Glutathione-Deficient Rabbits.\*

JEAN COLLINS-WILLIAMS AND C. CABELL BAILEY. (Introduced by Alexander Marble.)

*From the George F. Baker Clinic, New England Deaconess Hospital, Boston, Mass.*

The production of diabetes by the administration of alloxan or chemically related compounds, dialuric acid or alloxantin, has provoked considerable speculation whether these may occur in the body as normal or abnormal metabolites in sufficient concentration to damage the islets of Langerhans. Since alloxan can be obtained by the reduction of uric acid *in vitro*, Lazarow<sup>1</sup> suggests that an abnormal purine metabolism may possibly produce alloxan or an alloxan-like compound.

The injection of alloxan produces a pro-

found decrease in blood reduced glutathione in rabbits<sup>2</sup> and rats<sup>3</sup> and the administration of glutathione prior to the injection of alloxan prevents the diabetogenic action of the latter.<sup>1</sup> This suggests that glutathione may represent one of the natural protections of the body against alloxan or alloxan-like substances.

Griffiths<sup>4</sup> artificially lowered the blood glutathione in 4 rabbits from an average of 38 to 18-23 mg/100 cc by feeding a diet de-

\* This work was aided by grants from the American Cyanamid Co., and the Diabetic Fund.

<sup>1</sup> Lazarow, A., *Proc. Soc. Exp. Biol. and Med.*, 1946, 61, 441.

<sup>2</sup> Leech, R. S., and Bailey, C. C., *J. Biol. Chem.*, 1945, 157, 525.

<sup>3</sup> Bruckmann, G., and Wertheimer, E., *J. Biol. Chem.*, 1947, 168, 241.

<sup>4</sup> Griffiths, M., *J. Biol. Chem.*, 1948, 172, 823.

showed large quantities of the ink.

In control animals with both the celiac and superior mesenteric arteries occluded but no alloxan or ink injected the blood sugar remained within a normal range.

**Results.** Table I shows the blood sugar of 10 rabbits given alloxan just after clamping the celiac and superior mesenteric arteries.

An initial hyperglycemic phase usually seen after the injection of alloxan in normal animals was present but not striking in most of these experiments.

Six of the 10 rabbits developed severe hypoglycemia within 3 to 9 hours after alloxan with blood sugar values of 34 to 47 mg/100 cc and 2 others had blood sugar values of 59 and 69 respectively. The remaining 2 failed to develop hypoglycemia by 6 hours when they were fed and the possibility exists that hypoglycemia might have occurred had they been fasted longer.

Nine of the 10 animals failed to develop diabetes. Eight were sacrificed in 3 to 15 days and one rabbit, which had unusually prolonged hypoglycemia, died of an undetermined cause 2 days after the injection with a normal blood sugar. In the remaining rabbit (No. 5) the blood sugar was normal for 3 days, reached 171 mg on the 4th day and 258 and 230 respectively, on the 5th and 6th day at which time the animal was sacrificed. Such a delayed onset of hyperglycemia occurs occasionally in unoperated rabbits given alloxan but usually definite hyperglycemia develops within 24 to 48 hours.

For histological examination 2 animals were sacrificed at 3 days, 3 at 5 days, 2 at 12 days and 2 at 15 days. No significant findings were noted in the liver, spleen, adrenals or kidneys in any of the animals. Seven rabbits (Nos. 2, 3, 6, 7, 8, 9, 10) showed no definite histological changes in the pancreas. The tissues of one animal (No. 1) were inadvertently destroyed. In the remaining 2 (Nos. 4 and 5), mild alterations in the pancreas were found.

In rabbit No. 5 which developed delayed hypoglycemia the islets showed (1) an apparent reduction in the total number of beta cells, some islets consisting solely of alpha cells; (2) a definite variation in size of the nuclei of beta cells, with some large hyper-

chromatic forms; (3) a total of 4 mitotic figures in beta cells found in examination of 2 different sections; and (4) a pallor of the cytoplasm of the beta cells, suggesting degranulation but without definite vacuoles. Rabbit 4, which had a normal blood sugar and appeared well when sacrificed 3 days after alloxan, showed the same type of histological changes but to a considerably less degree.

It should be pointed out that the changes described above are in marked contrast to the massive necrosis of beta cells which usually follows a diabetogenic dose of alloxan. The findings do suggest a mild injury of the beta cells with evidence of regeneration.

**Discussion.** These experiments strongly suggest that alloxan diabetes is pancreatic in origin for we have not observed definite diabetes when the pancreatic blood supply was interrupted sufficiently to prevent detectable changes in the islets of Langerhans.

Although very mild, transitory, delayed hyperglycemia was observed in 2 rabbits with only the celiac artery occluded and no islet changes were seen, which appears contradictory, it must be emphasized that these animals were sacrificed at 19 and 21 days after the administration of alloxan and it is well known that slight pancreatic islet damage observed in the first 2 or 3 days after alloxan injection may not be visible several days later. Since very small amounts of India ink were present in the pancreas of 1 of 3 animals with both celiac and superior mesenteric arteries occluded, and since 1 rabbit developed delayed hyperglycemia accompanied by slight but definite pancreatic changes, it is not certain that clamping these 2 vessels always eliminates completely the blood supply to the pancreas.

From these experiments it is impossible to say that no alloxan reached the pancreas and that the hypoglycemic phase was extrapancreatic in origin. However, hypoglycemia was observed to follow the injection of alloxan without the subsequent development of diabetes and without detectable changes in the islets of Langerhans even in 2 animals whose pancreas was examined at 2 and 3 days when alloxan damage to the islets should be severe.

These experiments suggest that the hypo-

TABLE II.  
Effect of Hemorrhage on Blood Reduced Glutathione Concentration in Rabbits.

Rabbit No.	Duration of bleeding period (days)	Amt. of blood withdrawn (cc)	Hemoglobin, g/100 cc		Blood glutathione, mg/100 cc	
			Initial	Final	Initial	Final
H1	6	53	8.4	5.9	35	23
H2	3	78	9.8	5.2	38	19
H3	9	109	10.3	8.1	42	42
H4	11	312	9.8	3.8	33	26
H5	3	85	7.8	3.4	33	17
H6	2	122	9.6	4.1	38	20

sulphydryl-deficient diet as the animals showed anorexia, loss of weight, marked loss of hair, anemia and generalized weakness. Of 21 rabbits on the diet 9 died before the blood glutathione was lowered to 25 mg/100 cc. The effect of the diet on weight, hemoglobin and blood glutathione in the remaining 12 animals is shown in Table I. Only 5 of these rabbits (D1, 2, 6, 7, 10) showed a lowering of blood glutathione to 22 mg or less in 4 to 8 weeks of diet and 1 (D12) in 12 weeks. The remaining 6 were injected with uric acid although the blood glutathione was still 27 to 38 mg/100 cc after 9 to 16 weeks of the diet. Unless the blood glutathione dropped markedly in the first 4 to 8 weeks it seemed that it was unlikely to fall to 25 mg or less with a prolonged diet.

At the suggestion of Griffiths<sup>11</sup> 18 young rabbits weighing 0.5 to 1.0 kg were put on a modified diet containing one-half the protein of the original diet but none of these survived beyond 4 weeks. Six adult rabbits also failed to survive beyond 4 weeks on this reduced protein diet.

The effect of hemorrhage on the blood glutathione is shown in Table II. In 4 rabbits (H1, 2, 5, 6) there was a reduction to 23 mg/100 cc or less with removal of 53 to 122 cc of blood in 2 to 6 days but 2 others (H3,4) were apparently better able to compensate for the blood loss. This compensation has been observed in patients with anemia.<sup>12</sup> One of our rabbits showed no lowering of blood glutathione after 109 cc of blood were re-

moved in 9 days and in another there was only moderate lowering after removal of 312 cc in 11 days.

Table III shows the effect of intraperitoneal injections of uric acid into control rabbits and into those made sulphydryl-deficient by diet or bleeding. In each group, including the controls, most of the animals had apparent hyperglycemia for several hours after the uric acid injection but by 24 hours the blood sugar was normal. Such initial hyperglycemia did not occur in those animals which failed to show an elevation of the blood uric acid. In 2 animals (D6, C2) a second injection of uric acid at a later date gave the usual increase in blood uric acid accompanied by apparent hyperglycemia. By the Nelson sugar method which is less affected by a high blood uric acid, the hyperglycemia was less but in several cases quite definite.

After the first 24 hours, when the blood uric acid was always negative, one of the rabbits on the deficient diet (D6) showed a marked transitory hyperglycemia reaching 366 mg/100 cc 2 days after injection of uric acid and one other (D4) a mild hyperglycemia of 200 mg on the 5th day. In the hemorrhage group one rabbit (H4) showed a transient hyperglycemia after uric acid reaching 218 mg on the 3rd day. None of the other animals had blood sugar values significantly higher than the control group injected with uric acid.

Rabbit D6 which developed marked hyperglycemia at 2 days was an animal which had failed to show uric acid in the blood after it had been injected intraperitoneally. When D6 was reinjected 8 weeks later the blood

<sup>11</sup> Griffiths, M., personal communication.

<sup>12</sup> Pickard, R. J., and Marsden, C. S., *J. Lab. and Clin. Med.*, 1933, 19, 395.

TABLE I.  
Effect of a Cystine and Methionine Deficient Diet on Weight, Hemoglobin, and Blood Glutathione in Rabbits.

Rabbit No.	Weeks of diet	Wt, g		Hemoglobin, g/100 cc		Blood reduced glutathione, mg/100 cc	
		Initial	Final	Initial	Final	Initial	Final
D1	8	1759	1050	—	5.0	32	20
D2	8	1710	1410	—	5.0	45	22
(2)*	6	1700	1110	5.4	4.7	25	21
D3	11	1775	1222	—	5.1	46	27
D4	7	1952	1493	—	—	56	27
D5	9	1341	1275	—	6.2	46	28
D6	6	1767	1360	—	6.4	44	21
(2)*	7	2180	1280	9.4	7.1	43	17
D7	5	1720	1555	9.3	4.6	50	15
D8	16	1975	1250	11.3	3.8	49	29
D9	12	2480	1810	10.0	5.0	44	33
D10	4	3120	2640	8.8	7.8	44	19
D11	14	3210	1950	11.1	5.3	48	38
D12	12	2980	3015	10.6	7.9	38	19

\* After an interval of 1 to 2 weeks on usual rabbit pellet diet.

ficient in cystine and methionine. Uric acid, in dosage of 1 g/kg, was then injected intraperitoneally and the blood sugar determined. He reports that a moderate hyperglycemia ensued by the 2nd day after the injection and remained for 4-5 days. Thereafter normal blood sugar levels were regained.

This paper describes an attempt to repeat Griffiths' experiments and also to determine if decreasing the blood glutathione by bleeding renders the animal sensitive to the production of hyperglycemia when given uric acid.

**Methods and Materials.** Male albino rabbits weighing 1.3 to 3.2 kg were used and were fasted for 16 to 18 hours before the injection of uric acid.

Griffiths' modification<sup>4</sup> of a cystine and methionine deficient diet described by Haag and Wright<sup>5</sup> was used and this was fed to each animal for 4 to 16 weeks to lower the blood glutathione. An attempt was made to secure blood glutathione levels under 25 mg/100 cc before the injection of uric acid.

In the hemorrhage experiments varying amounts of blood were removed by repeated cardiac punctures. Approximately 30 cc were removed daily or almost daily and bleeding was continued until a low blood glutathi-

one was obtained in 5 rabbits. Uric acid was then injected.

Control rabbits on the usual laboratory diet of Park and Pollard rabbit pellets were also injected with uric acid.

Uric acid (Eastman Kodak or Coleman and Bell Co.) was administered intraperitoneally in dosage of 1 g/kg in a suspension in 20 cc of water. Blood sugar and blood uric acid concentrations were determined before and at intervals after the uric acid injection.

Blood reduced glutathione was determined by the Potter and Franke<sup>6</sup> modification of the Benedict and Gottschall<sup>7</sup> method adapted to the Leitz photoelectric colorimeter. Blood uric acid was determined by the method of Folin.<sup>8</sup> Blood sugar was determined by the Folin and Malmros<sup>9</sup> micromethod and in some cases, to decrease the reducing effect of the uric acid on the reagents, Nelson's<sup>10</sup> blood sugar method was used.

**Results.** It was found difficult to lower the blood glutathione in rabbits by feeding the

<sup>6</sup> Potter, V., and Franke, K., *J. Nutrition*, 1935, 9, 1.

<sup>7</sup> Benedict, S. R., and Gottschall, G., *J. Biol. Chem.*, 1932, 99, 729.

<sup>8</sup> Folin, O., *J. Biol. Chem.*, 1930, 86, 179.

<sup>9</sup> Folin, O., and Malmros, H. J., *J. Biol. Chem.*, 1929, 83, 115.

<sup>10</sup> Nelson, N., *J. Biol. Chem.*, 1944, 153, 375.

<sup>5</sup> Haag, J. R., and Wright, L. D., *J. Nutrition*, 1940, 19, 563.

uric acid rose to 31 mg/100 cc but no hyperglycemia ensued after 24 hours. Rabbit D4 with a mild hyperglycemia reaching 200 mg did show a blood uric acid elevation to 20 mg/100 cc after injection.

In one rabbit with blood glutathione lowered to 25 mg/100 cc, neutralized sodium urate in 1.2% solution was injected intravenously. Five injections of 0.4 mg/kg each were given in 15 days. The blood sugar remained normal throughout.

*Discussion.* The cystine and methionine deficient diet theoretically lowers the blood glutathione, a tripeptide containing glycine, glutamic acid and cysteine, by depriving the body of cystine. The rabbits in our experiment did not tolerate this diet well and it was therefore difficult to secure rabbits with lowered blood glutathione for uric acid injection.

No explanation is apparent why one animal showed marked hyperglycemia for 4 to 5 days after uric acid while others which had blood glutathione values as low or lower failed to do so. Only 2 of the 8 rabbits with glutathione of 27 mg or less showed hyperglycemia.

We cannot explain why only a trace or no blood uric acid could be detected after its intraperitoneal injection in 7 instances whereas others attained blood uric acid levels up to 41 mg/100 cc at the same time intervals, nor can we explain the different response in the same animal with a second injection.

A rough correlation existed in most cases between the apparent hyperglycemia observed in the first few hours after injection and the height of the blood uric acid, which may be explained in part by the fact that uric acid reduces the ferricyanide blood sugar reagent. However there were several instances of rising blood sugar with falling uric acid levels and also some high blood sugar values by the Nelson method.

Since all animals became quite anemic on the cystine and methionine deficient diet, and since almost all of the glutathione in normal

blood is in the red cell, attempts were made to lower the glutathione by repeated bleedings. In the small group of animals with the blood glutathione lowered in this manner transient hyperglycemia was observed at 3 to 4 days in 1 of 4 rabbits.

Although the results of these experiments were variable and in most cases negative it was thought worthwhile to report them in view of the current interest in glutathione in the prevention of diabetes and the possible role of uric acid or related compounds in the production of diabetes.

In spite of the large proportion of negative results the marked hyperglycemia in one rabbit and the moderate hyperglycemia in 2 others given uric acid with a lowered blood glutathione suggest that further investigation of this subject may be worthwhile. However the impression is gained that if uric acid under these conditions is diabetogenic, its action is very weak.

*Summary.* Attempts were made to confirm the report of Griffiths that transient diabetes follows the intraperitoneal injection of uric acid into rabbits in which the blood glutathione has been lowered by feeding a diet deficient in cystine and methionine.

In our experiment 21 rabbits were fed the deficient diet. Twelve animals surviving the dietary restriction were injected with uric acid although only 6 had a blood glutathione below 23 mg/100 cc. One of these had a definite transient hyperglycemia for 5 to 6 days after the uric acid injection. No blood sugar changes were seen in the other animals after 24 hours. One rabbit with a blood glutathione of 27 mg/100 cc showed a moderate transient hyperglycemia.

When the blood glutathione was lowered by bleeding instead of diet only one of 4 rabbits injected with uric acid showed a transient hyperglycemia after 24 hours.

It is concluded that if uric acid is diabetogenic under these conditions its action is weak.

TABLE III.  
Effect of Uric Acid Injection on the Blood Sugar in Rabbits.

No.	Group	Blood-glutathione when injected (mg/100 cc)	Highest blood uric acid noted after injection (mg/100 cc)	Blood sugar (mg/100 cc)												
				Hours					Time after uric acid							
				Fasting values					Non-fasting values†							
				0	1	3	5		1	2	3	4	5	6		
D1	Diet*	20	28	135	160	120	—	48 dead	102	149	147	165	137	137		
D2	"	22	41	128	205	235	214	102	149	147	165	137	137	137		
D3	(2)	21	28	124	246	260	218	124	181	118	139	139	139	139		
D4	Diet	27	trace	105	90	80	116	80	114	124	160	200	200	200		
D5	"	27	20	114	160	135	—	105	132	142	160	200	200	200		
D6	"	28	15	129	180	165	133	95	133	89	116	—	—	—		
D7	(2)	21	neg.	107	128	135	139	153	366	352	278	205	205	205		
D8	Diet	15	31	130	211	214	233	114	135	139	114	dead	125	125		
D9	"	15	trace	135	260	354	308	84 dead	87	103	95	102	—	—		
D10	"	29	10	103	260	354	308	84 dead	87	103	95	102	—	—		
D11	"	33	neg.	124	103	112	67	116	145	122	127	127	127	127		
D12	"	19	7	107	143	147	135	116	145	122	127	127	127	127		
H1	Hemorrhage	23	16	171	211 died	211 died	211 died	124	128	130	137	143	143	143		
H2	"	19	13	141	208	250	239	120	153	130	139	139	139	139		
H3	"	42	3	143	155	147	128	122	109	109	112	124	124	124		
H4	"	26	7	124	124	174	124	124	124	278	194	165	165	165		
H5	"	17	neg.	124	135	128	128	120	135	120	135	135	135	135		
H6	"	20	trace	116	128	114	112	133	135	130	130	130	130	130		
C1	Control†	57	11	85	200	165	169 dead	118	151	156	128	128	128	128		
C2	"	38	neg.	132	124	128	92	118	151	156	128	128	128	128		
C3	(2)	38	11	124	188	294	294	114	120	120	120	120	120	120		
C4	"	59	14	92	135	137	137	101	120	114	120	120	120	120		
C4	(2)	47	8	95	135	149	143	107	116	109	95	95	95	95		
C4	(2)	42	14	101	151	153	167	101 reinjected daily	101	101	101	101	101	101		

\* Cystine and methionine deficient diet.  
† Diet as in table.

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† Park and Pollard rabbit pellet diet.

‡ For convenience in interpretation, all blood sugar values of 160 mg or above at 1 day or later, appear in *italic* type.

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# 17265. Effect of Germination on Phytin Content and Phytase Activity of Some Common Indian Pulses.

SACHCHIDANANDA BANERJEE AND NIRMALENDU NANDI.

From the Department of Physiology, Presidency College, Calcutta, India.

Phosphorus present in foodstuffs as phytin is largely unavailable for nutrition. Studies have been made by several workers of the availability of phytin phosphorus. Krieger, Bunkfeldt and Steenbock<sup>1</sup> observed that when rats were fed a cereal ration of normal calcium content the utilization of phosphorus from phytin was markedly enhanced by the addition of vitamin D. Boutwell, Geyer, Halverson and Hart<sup>2</sup> studied the availability of wheat bran phosphorus, which contains about 85% of phosphorus as phytin, in rats and observed that an adequate intake of vitamin D increased the utilization of phytin phosphorus as measured by bone ash. Pringle and Moran<sup>3</sup> and Widdowson<sup>4</sup> reported that phytin in wheat meal or white flour is partly destroyed during the process of preparation of bread. Pulses are rich in phytin. The vitamin content of pulses is found to increase during the process of germination.<sup>5,6</sup> It was, therefore, considered desirable to investigate whether the phytin content of pulses is diminished during germination.

**Methods.** One gram of clean and dry seeds of pulses were placed in a washed and sterilized petri dish. The seeds in each dish were soaked daily with 1 cc of redistilled water for a period of 5 days. Phytin phosphorus content of the seeds was estimated both before and during the course of germination up to 5 days. Total phosphorus content

of the seeds was also estimated.

**Total phosphorus.** The seeds were finely ground and digested in a Kjeldahl flask with 10 cc of concentrated sulfuric acid and 1 cc of 60% perchloric acid for half an hour till the digestion was complete and the contents were diluted to a definite volume and the total phosphorus content was estimated by the method of Fiske and Subbarow.<sup>7</sup>

**Phytin phosphorus.** 1 g of the material was extracted with 50 cc of N/2 hydrochloric acid in a glass stoppered bottle for 2 hours and filtered. The phytin present in the filtrate was precipitated as the insoluble iron salt. The insoluble salt was converted to soluble sodium salt which was digested with sulfuric acid and perchloric acid mixture and the inorganic phosphate thus obtained was estimated by the method of Fiske and Subbarow.<sup>7</sup>

**Phytase activity.** 5 g of pulses, before or during the course of germination, were ground and extracted for 12 hours with 50 cc of water saturated with toluene. The extract was centrifuged. Inorganic phosphorus in a 10 cc portion of the centrifugate, representing 1 g of the pulse, was estimated and to another 10 cc of the extract was added acetate buffer of pH 5.2 and a substrate containing

TABLE I.  
Mg of Phytin Phosphorus Present in 100 g of Pulses.

Pulses	Days of germination					
	0	1	2	3	4	5
<i>Phaseolus mungo</i>	156	156	101	96	95	95
<i>Phaseolus radiatus</i>	158	138	117	110	109	109
<i>Cicer arietinum</i>	138	125	117	97	80	80
<i>Dolichos lablab</i>	138	127	114	104	105	105

<sup>7</sup> Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, 1925, **66**, 375.

<sup>1</sup> Krieger, C. H., Bunkfeldt, R., and Steenbock, H., *J. Nutrition*, 1940, **20**, 7.

<sup>2</sup> Boutwell, R. K., Geyer, A. P., Halverson, A. W., and Hart, E. B., *J. Nutrition*, 1946, **31**, 193.

<sup>3</sup> Pringle, W. J. S., and Moran, T., *J. Soc. Chem. Indust.*, 1942, **61**, 108.

<sup>4</sup> Widdowson, E. M., *Nature*, 1941, **148**, 219.

<sup>5</sup> Ahmad, B., Qureshi, A. A., Babbar, I., and Sawhney, P. C., *Ann. Biochem. Exp. Med.*, 1946, **6**, 29.

<sup>6</sup> French, C. E., Berryman, G. H., Goorley, J. T., Harper, H. A., Harkness, D. M., and Thacker, E. J., *J. Nutrition*, 1944, **28**, 63.

TABLE II

7 of Phosphorus Liberated from the Substrate Containing 10 mg of Phosphorus in the Form of Sodium Phytate by the Phytase Present in 1 g of the Pulse Either Before or After Germination.

Pulses	Days of germination					
	0	1	2	3	4	5
<i>Phaseolus mungo</i>	39	46	66	82	91	91
<i>Phaseolus radiatus</i>	167	179	200	206	208	208
<i>Cicer arietinum</i>	39	43	47	50	50	50
<i>Dolichos lablab</i>	172	186	191	192	191	192

10 mg of phosphorus as sodium salt of phytin. The mixture was incubated at 35°C for 6 hours. The phosphorus liberated from phytin was estimated by the method of Fiske and Subbarow.<sup>7</sup>

**Results.** The total phosphorus content in mg per 100 g of *phaseolus mungo*, *phaseolus radiatus*, *cicer arietinum* and *dolichos lablab* was respectively 375, 302, 219 and 247. Phytin phosphorus values and phytase activities of the pulses are given in Tables I and II.

**Discussion.** Phytin phosphorus values of all the 4 varieties of pulses studied were found to decrease gradually during the process of

germination and the maximum lowering of the value occurred either on the third or on the fourth day of germination. The phytase activity of the pulses was found to increase during the course of germination and when the phytin content was minimum the phytase activity was maximum. This suggests that the enzyme phytase which is formed during the process of germination acts upon the phytin phosphorus and partly destroys it. It has been observed by French *et al.*<sup>6</sup> that 48 hours after germination of peas there is a considerable increase in the inositol value of the pulse. Phytin being the calcium or magnesium salt of inositol hexaphosphoric acid, the increased inositol value of the pulse after germination might be due to the breaking down of phytin by the enzyme phytase. The germinated pulse is therefore nutritionally superior to the ungerminated one.

**Summary.** Phytin content and phytase activity of 4 pulses have been estimated both before and for varying periods after germination. Phytin content gradually diminished along with the increase in the phytase activity of the germinating pulse which was maximum either on the third or on the fourth day of germination.

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## 17266. Creatinine, Potassium, and Virus Content of the Muscles Following Infection with the "Coxsackie Virus."<sup>2</sup>

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From Division of Laboratories and Research, New York State Department of Health, Albany.

A number of specimens of feces from children exhibiting symptoms of poliomyelitis have yielded viruses that produce degeneration of the skeletal muscles of suckling mice and hamsters.<sup>1,2</sup> The anatomic response in man is not known but the clinical records of

patients from whose feces virus was isolated suggest that muscle weakness or paralysis is common and sometimes persists for months. While considering means of testing patients for muscle degeneration, it occurred to us that creatine losses probably accompany the destruction of the striated muscle cells and,

\* This agent is being called "Coxsackie Virus" since the first recognized human cases were residents of that New York village.

<sup>1</sup> Dalldorf, Gilbert, and Sickles, G. M., *Science*, 1948, 108, 61.

<sup>2</sup> Dalldorf, Gilbert, Sickles, G. M., Plager, Hildegard, and Gifford, Rebecca, *J. Exp. Med.*, 1949, 89, 567.

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<i>Dolichos lablab</i>	172	186	191	192	191	192

10 mg of phosphorus as sodium salt of phytin. The mixture was incubated at 35°C for 6 hours. The phosphorus liberated from phytin was estimated by the method of Fiske and Subbarow.<sup>7</sup>

**Results.** The total phosphorus content in mg per 100 g of *phaseolus mungo*, *phaseolus radiatus*, *cicer arietinum* and *dolichos lablab* was respectively 375, 302, 219 and 247. Phytin phosphorus values and phytase activities of the pulses are given in Tables I and II.

**Discussion.** Phytin phosphorus values of all the 4 varieties of pulses studied were found to decrease gradually during the process of

germination and the maximum lowering of the value occurred either on the third or on the fourth day of germination. The phytase activity of the pulses was found to increase during the course of germination and when the phytin content was minimum the phytase activity was maximum. This suggests that the enzyme phytase which is formed during the process of germination acts upon the phytin phosphorus and partly destroys it. It has been observed by French *et al.*<sup>6</sup> that 48 hours after germination of peas there is a considerable increase in the inositol value of the pulse. Phytin being the calcium or magnesium salt of inositol hexaphosphoric acid, the increased inositol value of the pulse after germination might be due to the breaking down of phytin by the enzyme phytase. The germinated pulse is therefore nutritionally superior to the ungerminated one.

**Summary.** Phytin content and phytase activity of 4 pulses have been estimated both before and for varying periods after germination. Phytin content gradually diminished along with the increase in the phytase activity of the germinating pulse which was maximum either on the third or on the fourth day of germination.

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## 17266. Creatinine, Potassium, and Virus Content of the Muscles Following Infection with the "Coxsackie Virus."\*

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A number of specimens of feces from children exhibiting symptoms of poliomyelitis have yielded viruses that produce degeneration of the skeletal muscles of suckling mice and hamsters.<sup>1,2</sup> The anatomic response in man is not known but the clinical records of

patients from whose feces virus was isolated suggest that muscle weakness or paralysis is common and sometimes persists for months. While considering means of testing patients for muscle degeneration, it occurred to us that creatine losses probably accompany the destruction of the striated muscle cells and,

\* This agent is being called "Coxsackie Virus" since the first recognized human cases were residents of that New York village.

<sup>1</sup> Dalldorf, Gilbert, and Sickles, G. M., *Science*, 1948, 108, 61.

<sup>2</sup> Dalldorf, Gilbert, Sickles, G. M., Plager, Hildgard, and Gifford, Rebecca, *J. Exp. Med.*, 1949, 89, 567.

TABLE I.  
Concentration of Total Creatinine in Urine of Normal and Infected Suckling Mice.

Group	Age (days)	Total creatinine					
		Normal animals		Infected animals			
		No. in group	mg/ml	No. in group	Days following inoculation	Paralysis	mg/ml
1	6	22	0.45	22	1	—	0.3
	7	20	0.50	24	2	+	1.5
	8	20	0.50	18	3	++	2.7
	9	10	0.60	3	4	++++	2.0
2	6	22	0.30	23	1	—	0.33
	7	22	0.25	23	2	—	1.01
	7½	22	0.47	22	2½	+	3.00
	8	19	0.40	21	3	++	3.00
	8½	18	0.25	14	3½	+++	2.72
	9	17	0.26	8	4	++++	2.38
3	7	16	0.22	18	1	—	0.81
	7½	16	0.29	17	1½	—	1.05
	8	16	0.05	18	2	+	1.92
	8½	16	0.45	18	2½	++	3.50
	9	16	0.30	18	3	+++	3.50

further, that, since the muscle weakness in some patients was migratory and transient, serum potassium concentration should be tested. It has been impossible to secure suitable serum specimens from immature mice but the potassium and total creatinine values of the muscles have been determined, as well as urinary creatinine. The present report of these results includes, in addition, certain observations on the infectivity of the muscles.

**Methods.** The muscle samples for chemical analyses taken from immature mice at various times following intraperitoneal injection of brain suspensions of the T. T. strain, were placed in tared and sealed containers and weighed within 2 hours. The urine samples were obtained by lightly pressing on the lower abdomen with a wooden applicator. Since suckling mice do not urinate without external stimulus, small urine specimens were readily procured. In order to avoid possible differences between litters, the mice were first pooled and redistributed at random<sup>3</sup> before inoculation.

Potassium was determined by the phosphotungstate method of Van Slyke and Rieben.<sup>4</sup>

The samples for muscle creatinine determinations were prepared according to the directions of Rose, Helmer, and Chanutin,<sup>5</sup> and creatinine was determined by the colorimetric method of Folin and Wu.<sup>6</sup>

Excised muscle fragments were initially used for infectivity tests. Subsequently, preparations consisting of pools of the extremities, including bone and fascia, were used.

**Potassium and Creatinine Determinations.** Potassium determinations were made of samples of muscle from 24 normal and 23 paralyzed suckling mice. The mean for the first group was  $2.96 \pm 0.16$  mg per g of tissue, and that of the experimental group was  $2.08 \pm 0.28$  mg per g. The number following the  $\pm$  sign is the mean deviation. The greater value for the experimental group may be due to the uneven distribution of the muscle lesions. A striking feature of this experience was that all but one of the potassium values of the paralyzed animals were less than the least value of the controls.

Creatinine determinations were made of 8 samples of each of the above groups. The

<sup>5</sup> Rose, W. C., Helmer, O. M., and Chanutin, Alfred, *J. Biol. Chem.*, 1927, **75**, 543.

<sup>6</sup> Folin, Otto, and Wu, Hsien, *J. Biol. Chem.*, 1919, **38**, 81.

<sup>3</sup> Thompson, W. R., *Bact. Rev.*, 1947, **11**, 115.

<sup>4</sup> Van Slyke, D. D., and Rieben, W. K., *J. Biol. Chem.*, 1944, **156**, 743.

TABLE II.  
Infectivity of Pools of Legs and of Brains of Paralyzed Mice.

Source of virus	Test animals	Dilutions of virus								
		10-1	10-2	10-3	10-4	10-5	10-6	10-7	10-8	10-9
Suckling mice legs	Suckling mice 4-5 days				8/8	8/8	8/8	7/8	0/7	1/7
" " "	Weanling mice 7-8 g	9/9	8/10	3/10	1/10					
" " "	" " 8-10 g	0/10	0/10	0/10	0/10					
" " "	Mice 10-12 g	0/5								
" " "	" 14 g	0/5								
Suckling mice brains	Suckling mice 4-5 days	8/8	8/8	4/8	0/7	0/7				
" " "	Weanling mice 7-8 g	1/10	1/10	1/10	0/10	1/10				
Weanling mice legs	Suckling mice 7 days				7/7	8/8	7/7	4/8		
" " "	Weanling mice 7-8 g	7/7	4/7	2/7	2/7					
Weanling mice brains	Suckling mice 7 days	7/7	8/8	2/7	0/7					
" " "	Weanling mice 7-8 g	0/7	0/7	0/7	0/7					

Note: No. of mice paralyzed or dead/No. of mice inoculated.

mean concentration of total creatine in the control group was  $1.95 \pm 0.15$  mg per g and for the experimental group,  $0.96 \pm 0.23$  mg per g.

The concentration of total creatinine (creatinine plus creatinine) in the urine of various normal and infected suckling mice is shown in Table I. The mean for the normal animals was 0.35 mg per ml, while the concentration in diseased animals was frequently ten times as great. Increased excretion of creatinine occurred in two instances before the onset of symptoms. It should be noted that the samples from the sick mice were smaller. This is thought to have been due to the smaller size of the affected animals, since a comparison of their urinary pigments did not indicate that their urine was more concentrated. Stunting is characteristic of suckling mice infected with the virus. Whether this is a result of intrinsic changes or inferior nursing we do not know.

**Infectivity of muscle.** The muscles of paralyzed mice are more infectious than the tissues of the central nervous system. Suspensions prepared from pooled legs are roughly ten thousand times as infectious for suckling mice as those prepared from brains (Table II). When 10- to 12-g mice are inoculated with brain suspensions the organs contain little or no virus and while an occasional focus of muscle degeneration may sometimes be found, the mice exhibit no recognizable signs of infection. On the other hand, 10- to 12-g mice inoculated with muscle suspensions are some-

times paralyzed and weanling mice (7 to 8 g) are usually paralyzed. These observations prompted us to attempt to adapt the virus to 10- to 12-g mice by alternate passage<sup>7-10</sup> through suckling mice. Six alternate passages have shown no increase in infectivity and the brains and muscles of the older mice remain less infectious than those of suckling animals. Serial passages in older mice have also failed.

The greater infectivity of muscle suspensions provided effective antisera from recovered hamsters and a rhesus monkey. The monkey showed no signs of infection following intramuscular inoculation but developed humoral antibodies after a series of intraperitoneal injections. Paralysis and muscle lesions were induced in one of 6 new-born guinea pigs inoculated intraperitoneally with 0.5 ml of a 10% suspension of hamster legs. The paralyzed extremity was infective for suckling mice, while the brain was not.

Virus has been found in the feces of 7- to 8-g mice on the day of paralysis but not in the feces of 18-day-old hamsters that had been paralyzed for 2 days.

Limited tests with a few of the strains of

<sup>7</sup> Baker, J. A., *Amer. J. Vet. Res.*, 1946, **7**, 179.

<sup>8</sup> Baker, J. A., *Proc. Soc. Exp. Biol. and Med.*, 1946, **63**, 183.

<sup>9</sup> Koprowski, Hilary, James, T. R., and Cox, H. R., *Proc. Soc. Exp. Biol. and Med.*, 1946, **63**, 178.

<sup>10</sup> Dean, D. J., and Dalldorf, Gilbert, *J. Exp. Med.*, 1948, **88**, 645.

virus that affect both central nervous system and muscle have shown that suspensions of legs are either less virulent or about as virulent as suspensions of brains. This conforms with the signs of infection, with the histologic findings, and with the degree of creatinuria associated with the two types of disease.

*Discussion.* Whether or not these observations have application in clinical medicine is unknown. Creatinuria follows paralytic poliomyelitis<sup>11-13</sup> but has been assumed to be a sequel of neurone destruction. Since there is some evidence that muscle degeneration may occur in the initial stages of the disease,<sup>14</sup> the subject should be re-examined. Hassin described a case of poliomyelitis of the Landry type in which the lesions were restricted to the muscles.<sup>15</sup> Clawson is reported to have found no muscle lesions in a series of 22 cases of acute poliomyelitis<sup>16</sup> but Dr. Kornel Terplan, Buffalo General Hospital Laboratory,

<sup>11</sup> Gros, W., *Z. f. klin. Med.*, 1933, **126**, 152.

<sup>12</sup> Magers, E. J., *J. Biol. Chem.*, 1934, **105**, lvi. (*Sci. Proc.*).

<sup>13</sup> Wang, Erling, *Acta Med. Scand.*, 1939, supp. 105, p. 1.

<sup>14</sup> Carey, E. J., Massopust, L. C., Zeit, W., and Haushalter, E., *J. Neuropath. and Exp. Neurol.*, 1944, **3**, 121.

<sup>15</sup> Hassin, G. B., *J. Neuropath. and Exp. Neurol.*, 1943, **2**, 293.

provided us with histologic preparations from a case of fatal bulbar poliomyelitis in which extensive muscle lesions similar to those which occur in suckling mice were seen.

The infectivity of the muscles is consistent with the morphologic changes and reaffirms the peripheral nature of the paralysis in the mice. It is interesting that the higher titer of the muscle preparations makes it possible to induce paralysis in mice of an age-group that is resistant to infection with brain suspension. While these findings modify, they also confirm the original observation of the remarkable natural immunity of mice and hamsters following weaning. This is further supported by failure to adapt the T.T. strain to weaned mice by alternate passage through susceptible animals.

*Summary.* Infection of unweaned mice with certain strains of "Coxsackie virus" is followed by loss of muscle potassium and creatinine and by creatinuria.

The muscles of paralyzed mice are highly infectious.

<sup>16</sup> Bell, E. T., *The Progressive Pathology of Poliomyelitis*. In: *Poliomyelitis. Papers and Discussions presented at the First International Poliomyelitis Conference*. J. B. Lippincott, Philadelphia, 1949, p. 135.

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## 17267. The Role of Complement in the Lysis of Leucocytes by Tuberculo-protein.\*

JOSEPH M. MILLER,<sup>†</sup> JOHN H. VAUGHAN,<sup>‡</sup> AND CUTTING B. FAVOUR.  
(Introduced by J. H. Mueller.)

*From the Medical Clinics, Peter Bent Brigham Hospital, and the Department of Medicine, Harvard Medical School.*

The nature of the mechanism underlying delayed or tuberculin-type hypersensitivity has long interested students of immunology. Whereas many workers have attempted to demonstrate an antibody in tuberculin allergy

which was similar in properties and mode of action to antibodies known to be responsible for anaphylactic or Arthus type of hypersensitivity, the preponderance of such studies has led to the assumption that cells, rather than serum antibodies, were the mediators of tuberculin hypersensitivity. Holst<sup>1</sup> first noted

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<sup>†</sup> Post-doctorate Research Fellow, U.S.P.H.S.

<sup>‡</sup> Stanley W. Tausend Fellow in Medicine.

<sup>1</sup> Holst, P. M., *Tubercle*, 1922, **3**, 337.

decreased phagocytic activity and loss of differentiation between the nucleus and cytoplasm when tuberculin was added to leucocytes derived from tuberculous animals but no deviation from normal when horse serum was added to leucocytes from guinea pigs sensitized to horse serum. Similarly Stewart *et al.*<sup>2</sup> observed that tuberculin killed leucocytes derived from the tuberculous guinea pig while the leucocytes of a guinea pig sensitized to crystalline egg white were not affected by the addition of that specific antigen.<sup>3</sup> These and other studies<sup>4-9</sup> have distinguished this cytotoxic role of antigen on cells of tuberculin-type allergic animals from the harmless response of cells to antigen in the anaphylactic forms of hypersensitivity. In the latter instance, serum antibody is felt to be the crucial factor in the hypersensitivity manifestations. The failure to transfer passively tuberculin sensitivity by means of the serum of a sensitized animal and the successful transfer of such sensitivity with cells of the tuberculous guinea pig by Chase<sup>10</sup> and others<sup>11-13</sup> have given further support to the concept that cells, rather than serum antibody, were the prime mediators of tuberculin type hypersensitivity.

The studies of Favour,<sup>14</sup> using a one-hour period of *in vitro* observation, have provided

<sup>2</sup> Stewart, F. W., Long, P. H., and Bradley, J. L. *Am. J. Path.*, 1926, **2**, 47.

<sup>3</sup> Long, P. H., and Stewart, F. W., *Am. J. Path.*, 1926, **2**, 91.

<sup>4</sup> Rich, A. R., and Lewis, M. R., *Proc. Soc. Exp. Biol. and Med.*, 1927-28, **25**, 596.

<sup>5</sup> Meyer, K., and Loewenthal, H., *Z. f. Immunitätsforsch. u. exp. Therap.*, 1927, **54**, 420.

<sup>6</sup> Rich, A. R., and Lewis, M. R., *Bull. Johns Hopkins Hosp.*, 1932, **50**, 115.

<sup>7</sup> Aronson, J. D., *J. Immunol.*, 1933, **25**, 1.

<sup>8</sup> Moen, J. K., and Swift, H. F., *J. Exp. Med.*, 1936, **64**, 339.

<sup>9</sup> Heilman, D. H., Feldman, W. H., and Mann, F. C., *Am. Rev. Tub.*, 1944, **50**, 334.

<sup>10</sup> Chase, M., *Proc. Soc. Exp. Biol. and Med.*, 1945, **59**, 134.

<sup>11</sup> Cummings, M. M., Hoyt, M., and Gottshall, R. Y., *Public Health Rep.*, 1947, **62**, 994.

<sup>12</sup> Kirchheimer, W. F., and Weiser, R. S., *Proc. Soc. Exp. Biol. and Med.*, 1947, **66**, 166.

<sup>13</sup> Stavitsky, A. B., *Proc. Soc. Exp. Biol. and Med.*, 1948, **67**, 225.

a simpler method of determining the cytotoxicity of tuberculin and subsequent investigation in this laboratory has aimed at elucidating the mechanism of tuberculin allergy by this *in vitro* technic. Earlier work<sup>15</sup> with white cells of tuberculous humans indicated that tuberculin was specifically toxic to these cells. It was further assumed that complement was essential for such cytolysis since no tuberculin cytolysis was observed if the diluting serum was heated at 56°C for 30 minutes.

A more recent report from this laboratory,<sup>16</sup> however, has shown that it is unnecessary to use "sensitized tuberculous cells"<sup>§</sup> to demonstrate short term tuberculin cytolysis. *In vitro* lysis of leucocytes by tuberculo-protein has been found to be dependent on the presence of tuberculous plasma (or serum). Further studies<sup>17</sup> now make it apparent that the active component of tuberculous plasma is a heat labile globulin. This finding of an antibody-like factor in tuberculous plasma (or serum) which is responsible for the cytotoxic action of tuberculin on tuberculous as well as on normal leucocytes<sup>||</sup> prompted a reinvestigation of the role of complement in this reaction. Because of the heat lability of the plasma factor, it was necessary to resort to other methods of removing complement completely from tuberculous plasma (or serum).

*Experimental.* As in previous experiments,<sup>16-17</sup> white blood cells from a normal, healthy tuberculin-negative subject were separated, washed 3 times in saline and used as the target cell for determining tuberculin

<sup>14</sup> Favour, C. B., *Proc. Soc. Exp. Biol. and Med.*, 1947, **65**, 269.

<sup>15</sup> Fremont-Smith, P., and Favour, C. B., *Proc. Soc. Exp. Biol. and Med.*, 1948, **67**, 502.

<sup>16</sup> Miller, J. M., Favour, C. B., Wilson, B. A., and Umbarger, M. A., *Proc. Soc. Exp. Biol. and Med.*, 1949, **70**, 738.

<sup>§</sup> White cells from a patient having active tuberculosis.

<sup>17</sup> Miller, J. M., Favour, C. B., Wilson, B. A., and Umbarger, M. A., *Proc. Soc. Exp. Biol. and Med.*, 1949, **71**, 287.

<sup>||</sup> Cells from a normal, healthy, tuberculin-negative subject.



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<sup>1</sup> Holst, P. M., *Tubercle*, 1922, 3, 337.

TABLE I.  
Effect of Complement on *in vitro* Lysis of Normal Leucocytes by Tuberculin in Presence of Normal and Tuberculous Plasma.

	Complement															
	50% hemolytic units per ml															
Normal cells	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Tuberculous plasma	0.3	0.3	0.3	0.3												
Tuberculous plasma "decomplemented"*	0				0.6	0.6	0.6	0.6	0.6							
Normal plasma	234															
Normal plasma "decomplemented"*	0							0.3	0.3	0.3	0.3	0.3	0.6	0.6	0.6	0.6
Fresh type AB serum	214														0.1	0.1
Tuberculin antigen		0.1		0.1								0.1		0.1		0.1
Saline		0.1	0.2			0.1	0.1	0.2		0.1	0.1	0.2	0.1	0.1	0.2	0.1
Total WBC		9,650	9,330	9,770	9,330	6,270	6,330	6,130	6,260	10,110	10,190	7,725	7,320	5,400	5,480	6,120
5 min.		7,670	9,260	7,790	9,200	6,300	6,310	4,930	6,330	10,060	10,280	7,650	7,270	5,330	5,470	6,080
60 min.																
% Decrement		-20.5	-0.7	-20.2	-1.4	+0.5	-0.5	-19.5	+0.7	-0.5	+0.7	-0.8	-0.7	-1.2	-0.5	-0.5

\* To remove complement from 2 ml of plasma, 1 ml of a 1:1000 solution of crystalline bovine albumin and 1 ml of anti-bovine albumin rabbit serum was added; thus, final dilution of plasma was 1:2.

cytolysis, thus leaving the tuberculin antigen, the tuberculous plasma factor and complement as the 3 vital determinants of eventual cell breakdown. Plasma (or serum) was obtained from patients hospitalized for active pulmonary tuberculosis whose plasma had previously been shown to contain the active plasma factor. Plasma from normal, healthy tuberculin-negative donors was used as a control. In order to remove complement completely from this plasma without inactivating the heat-labile plasma factor essential for cell lysis, two separate antigen-antibody systems were used. In the first instance, pneumococcal type III polysaccharide and antipneumococcal type III rabbit antiserum (previously found to produce a precipitate with antigen diluted 1:2,000,000)<sup>†</sup> were added to tuberculous plasma and normal plasma in such dilution as to give a precipitate in the zone of equivalence for this antigen-antibody system and thus fix complement. This mixture was incubated at 37°C in a water bath for 2 hours and then in the ice box at 10°C for 2 days. As a second complement-fixing antigen-antibody system, crystalline bovine albumin and anti-bovine albumin rabbit serum (previously found to produce a precipitate with antigen diluted 1:800,000)<sup>†</sup> was similarly added to tuberculous and normal plasma so as to yield precipitate in the zone of equivalence for that system. Complement titers were determined on the untreated tuberculous and normal plasma as well as on such plasma to which had been added the pneumococcal polysaccharide and bovine albumin systems in order to confirm the complete absence of complement from the latter two systems.

In estimating the amount of complement present in individual untreated sera, a modification of the method proposed by Mayer *et al.*<sup>18</sup> was employed. Using 0.5 ml amounts of 4% sheep cell suspensions, sensitized with four units of high titer amboceptor, as the indicator system, the amount of

serum necessary to produce 50% hemolysis was determined. The final titer was calculated from the equation

$$C = \frac{D}{V} \times 2.5$$

where C = the number of 50% hemolytic units of complement present per ml of undiluted serum, D = the reciprocal of the dilution of serum used, V = the volume of this dilution necessary for 50% hemolysis, and 2.5 is a factor used in this laboratory to make the results referable to those of our determinations which use a system employing only 0.2 ml of sensitized sheep cells. By this method normal human sera usually have complement titers in the vicinity of 300 units per ml. Plasma may show slightly lower values because of the anticoagulant used. All dilutions were made with a veronal buffered saline containing optimal quantities of calcium and magnesium ions.<sup>19</sup>

In those sera from which complement had been removed, a titration procedure designed to detect complement sufficient to produce 50% hemolysis was obviously not applicable. It was necessary to detect minimal hemolysis. Accordingly, to 0.2 ml suspensions of sensitized sheep cells were added the test sera in dilutions just sufficient to abolish non-specific hemolysis and/or anticomplementary factors. The final volume was 0.8 ml. After incubation for 40 minutes at 37°C the cells were centrifuged and the supernatant examined for evidence of hemolysis. In no instance, with either of the complement-fixing antigen-antibody systems used, was there any demonstrable complement activity remaining in the tuberculous or normal sera.

In order to demonstrate that the cytolysis promoting capacity of such plasma could be restored by the readdition of complement, freshly drawn type AB serum from a tuberculin-negative subject was titered for complement and added to the "decomplemented" plasma in separate tubes. As the tuberculin antigen in all experiments, Old Tuberculin,

<sup>†</sup> Kindly supplied by M. H. Kaplan of the Department of Bacteriology, Harvard Medical School.

<sup>18</sup> Mayer, M. M., Eaton, B. B., and Heidelberger, M., *J. Immunol.*, 1946, **53**, 31.

<sup>19</sup> Mayer, M. M., Oster, A. G., and Heidelberger, M., *J. Exp. Med.*, 1946, **84**, 535.

TABLE L  
Effect of Complement on *in vitro* Lysis of Normal Leucocytes by Tuberculin in Presence of Normal and Tuberculous Plasma.

	Complement 50% hemolytic units per ml															
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Tuberculous plasma	217	0.3	0.3	0.3	0.3											
Tuberculous plasma "decomplemented"*	0				0.6	0.6	0.6	0.6	0.6							
Normal plasma	234							0.3	0.3	0.3	0.3	0.3	0.3			
Normal plasma "decomplemented"*	0												0.6	0.6	0.6	0.6
Fresh type AB serum	214		0.1	0.1			0.1	0.1	0.1				0.1	0.1	0.1	0.1
Tuberculin antigen		0.1	0.1		0.1		0.1		0.1		0.1		0.1		0.1	
Saline		0.1	0.2		0.1	0.1	0.2		0.1	0.1	0.2		0.1	0.1	0.2	0.1
Total WBC		9,450	9,330	9,770	9,330	6,270	6,330	6,130	6,260	10,110	10,130	7,725	7,320	5,400	5,480	6,120
5 min.		7,670	9,260	7,790	9,200	6,300	6,310	4,930	6,330	10,060	10,280	7,650	7,270	5,330	5,470	6,080
60 min.																
% Decrement		-20.5	-0.7	-20.2	-1.4	+0.5	-0.5	-19.5	+0.7	-0.5	+0.7	-0.8	-0.7	-1.2	+0.5	-0.5

\* To remove complement from 2 ml of plasma, 1 ml of a 1:1000 solution of crystalline bovine albumin and 1 ml of anti-bovine albumin rabbit serum was added; thus, final dilution of plasma was 1:2.

cytolysis, thus leaving the tuberculin antigen, the tuberculous plasma factor and complement as the 3 vital determinants of eventual cell breakdown. Plasma (or serum) was obtained from patients hospitalized for active pulmonary tuberculosis whose plasma had previously been shown to contain the active plasma factor. Plasma from normal, healthy tuberculin-negative donors was used as a control. In order to remove complement completely from this plasma without inactivating the heat-labile plasma factor essential for cell lysis, two separate antigen-antibody systems were used. In the first instance, pneumococcal type III polysaccharide and antipneumococcal type III rabbit antiserum (previously found to produce a precipitate with antigen diluted 1:2,000,000)<sup>†</sup> were added to tuberculous plasma and normal plasma in such dilution as to give a precipitate in the zone of equivalence for this antigen-antibody system and thus fix complement. This mixture was incubated at 37°C in a water bath for 2 hours and then in the ice box at 10°C for 2 days. As a second complement-fixing antigen-antibody system, crystalline bovine albumin and anti-bovine albumin rabbit serum (previously found to produce a precipitate with antigen diluted 1:800,000)<sup>†</sup> was similarly added to tuberculous and normal plasma so as to yield precipitate in the zone of equivalence for that system. Complement titers were determined on the untreated tuberculous and normal plasma as well as on such plasma to which had been added the pneumococcal polysaccharide and bovine albumin systems in order to confirm the complete absence of complement from the latter two systems.

In estimating the amount of complement activity present in individual untreated sera, a modification of the method proposed by Mayer *et al.*<sup>18</sup> was employed. Using 0.5 ml amounts of 4% sheep cell suspensions, sensitized with four units of high titer amboceptor, as the indicator system, the amount of

serum necessary to produce 50% hemolysis was determined. The final titer was calculated from the equation

$$C = \frac{D}{V} \times 2.5$$

where C = the number of 50% hemolytic units of complement present per ml of undiluted serum, D = the reciprocal of the dilution of serum used, V = the volume of this dilution necessary for 50% hemolysis, and 2.5 is a factor used in this laboratory to make the results referable to those of our determinations which use a system employing only 0.2 ml of sensitized sheep cells. By this method normal human sera usually have complement titers in the vicinity of 300 units per ml. Plasma may show slightly lower values because of the anticoagulant used. All dilutions were made with a veronal buffered saline containing optimal quantities of calcium and magnesium ions.<sup>19</sup>

In those sera from which complement had been removed, a titration procedure designed to detect complement sufficient to produce 50% hemolysis was obviously not applicable. It was necessary to detect minimal hemolysis. Accordingly, to 0.2 ml suspensions of sensitized sheep cells were added the test sera in dilutions just sufficient to abolish non-specific hemolysis and/or anticomplementary factors. The final volume was 0.8 ml. After incubation for 40 minutes at 37°C the cells were centrifuged and the supernatant examined for evidence of hemolysis. In no instance, with either of the complement-fixing antigen-antibody systems used, was there any demonstrable complement activity remaining in the tuberculous or normal sera.

In order to demonstrate that the cytolysis promoting capacity of such plasma could be restored by the readdition of complement, freshly drawn type AB serum from a tuberculin-negative subject was titered for complement and added to the "decomplemented" plasma in separate tubes. As the tuberculin antigen in all experiments, Old Tuberculin,

<sup>†</sup> Kindly supplied by M. H. Kaplan of the Department of Bacteriology, Harvard Medical School.  
<sup>18</sup> Mayer, M. M., Eaton, B. B., and Heidelberger, M., *J. Immunol.*, 1946, **53**, 31.

<sup>19</sup> Mayer, M. M., Oster, A. G., and Heidelberger, M., *J. Exp. Med.*, 1946, **84**, 535.

of *in vitro* tuberculin hypersensitivity has been further elucidated.

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ance of Mrs. Merle Umbarger, Mrs. Barbara Wilson, and Miss Elizabeth Geiler.

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## 17268. Alloxan Subdiabetes in Rabbits Detected by Modification of Glucose Tolerance by Adrenal Cortex Extract.

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The subdiabetic state may be defined as a phase of pancreatic diabetes characterized by subtle metabolic disorders; these disorders, in contrast to overt diabetes, are unaccompanied by hyperglycemia or glycosuria, and, in contrast to latent diabetes, are not accompanied by impaired glucose tolerance as determined by conventional methods. The existence of such a state, which might or might not be a prediabetic state, has been suspected by many students of human diabetes mellitus.

Although latent diabetes has been demonstrated in various animals,<sup>1-3</sup> subdiabetes and prediabetes have only been demonstrated by retrospective analysis of the records of mothers of overweight babies<sup>4</sup> and of the records of partially pancreatectomized animals.<sup>5,6</sup> The present studies were undertaken in an attempt to produce experimental subdiabetes, and, by exploiting the adrenal cortex-insulin antagonism,<sup>7</sup> to devise a method for its detection.

*Methods and Materials.* Mongrel and white

rabbits weighing 2.5-3.6 kg were used. Their diet consisted of oats, hay, carrots, cabbage, lettuce, and water *ad lib*. The animals were starved for 18-20 hours prior to each experimental procedure, including the alloxan injections. Sugar was determined on ear vein blood by the method of Hagedorn and Jensen;<sup>8</sup> no anticoagulant was used, and the precipitated samples were all permitted to stand, without fluoride, until the end of the third hour, when the determinations were made. Urinary sugar was not followed.

Glucose tolerance was tested by the intravenous injection, during about one minute, of 3 g of glucose in 50% solution, regardless of weight; this was followed by 2-3 cc of physiologic saline. Fasting, 1, 2, and 3 hour blood samples were drawn. The effect of adrenal cortical hormones on the glucose tolerance was studied by injecting aqueous adrenal cortex extract (Upjohn) intramuscularly,  $\frac{1}{2}$  cc/100 g body weight, one half hour before the glucose injection; the entire procedure will be called the *A. C. Tolerance Test*. It should be noted that the fasting blood sugar specimens in all A. C. tolerance tests were drawn one half hour after the injection of adrenal cortex extract (A.C.).

One day or more after the completion of preliminary tolerance tests, alloxan monohydrate (Eastman) was rapidly injected into the ear veins of fasted rabbits in 2.5% solution; further tolerance tests were performed, and subsequent doses of alloxan given, if indi-

<sup>1</sup> Shipley, E. G., and Rannefeld, A. N., *Endocrinology*, 1945, **37**, 313.

<sup>2</sup> Marinetti, R., and Andreani, G., *Boll. de Soc. Ital. di Biol. Sper.*, 1946, **22**, 860.

<sup>3</sup> Houssay, B. A., Brignone, R. F., Cardeza, A. F., and Sara, J., *Rev. Soc. Argent. de Biol.*, 1946, **22**, 241.

<sup>4</sup> Miller, H. C., *New England J. Med.*, 1945, **233**, 376.

<sup>5</sup> DeRobertis, E., *Rev. Soc. Argent. de Biol.*, 1945, **21**, 273.

<sup>6</sup> Bell, E. T., *Experimental Diabetes Mellitus*, Springfield, 1948.

<sup>7</sup> Cori, C., *The Harvey Lecture Series*, 1945-6, Lancaster, Pa., 1946.

<sup>8</sup> Hawk, P. B., Oser, B. L., and Summerson, W. H., *Practical Physiological Chemistry*, 12th ed., Philadelphia, Pa., 1947.

prepared as described earlier,<sup>20</sup> was used. From previous experiments, it was found best to use cells and plasma from homologous blood groups.

The methods of preparing and washing white cell concentrates, diluting these cells in the various plasma (or sera) and cell counting to estimate cytolysis were the same as described previously.<sup>16,21</sup>

**Results.** The results of a typical experiment are recorded in Table I. In this instance, complement was removed by means of the crystalline bovine albumin system but identical findings were observed with the pneumococcal polysaccharide antigen-antibody system.

1. As reported earlier,<sup>16</sup> a portion of thoroughly washed white blood cells from a healthy, tuberculin negative subject undergoes significant cytolysis when incubated for one hour at 37°C with freshly drawn tuberculous plasma and tuberculin antigen. No such cytolysis occurs if the plasma of a tuberculous patient is replaced by the plasma of a tuberculin-negative subject.

2. Removal of all complement from tuberculous plasma by means of an unrelated complement-fixing antigen-antibody system results in the loss of the ability of such plasma to affect cytolysis of white blood cells. The successful removal of all complement by this technic has been confirmed by the sheep cell hemolysis method described above. It can be noted that 0.6 ml of "decomplemented" plasma was used instead of the 0.3 ml used for fresh plasma. This was done since the complement fixing procedure resulted in a 1:2 dilution of the original plasma.

3. The addition to the system of active complement in the form of freshly drawn type AB tuberculin-negative serum restored completely the capacity of "decomplemented" tuberculous plasma to cause white cell destruction in the presence of Old Tuberculin.

4. Plasma from a tuberculin negative subject continued to have no effect on white blood

cells even after the addition of fresh complement.

5. The addition of further complement of known titer to this system in the form of freshly drawn AB serum from a tuberculin-negative donor does not further enhance the tuberculin cytolysis of this system. It may be assumed, therefore, that the tuberculous plasma already has adequate amounts of complement.

**Discussion.** The present report further elucidates the mechanism of the *in vitro* cytolysis of human white blood cells by tuberculo-protein. Contrary to the original hypothesis in this laboratory that tuberculin cytolysis was a direct toxic effect of tuberculin on the tuberculous "sensitized" cell, a more recent report<sup>16</sup> has shown that even normal white cells of a tuberculin negative donor can be lysed by tuberculin provided a factor in tuberculous plasma is also present. Various properties<sup>17</sup> of this factor suggest that it is a globulin. This work further characterizes such *in vitro* cytolysis as a manifestation of an antigen-antibody inter-action. It now appears evident that complement too is an essential component of this system.

The question of most significance in these studies is whether or not such *in vitro* short term tuberculin cytolysis, dependent as it is on antigen, antibody and complement, may be taken to be a valid analogue of *in vivo* cutaneous tuberculin hypersensitivity. If this be so, then the dependence of the antigen-antibody reaction upon complement has significance in indicating the role of the lytic action of this substance in this type of hypersensitivity. The successful passive transfer of tuberculin hypersensitivity in guinea pigs by Chase,<sup>18</sup> using living cells, allows the possibility that the hypersensitivity developed in the recipient animal may be due primarily to elaboration by the cells of antibody which is identical in properties to the plasma factor demonstrated in this laboratory.

**Summary.** The essential role of complement in the *in vitro* cytolysis of human white blood cells by tuberculin has been demonstrated, using two different systems of complement removal. In this way, the mechanism

<sup>20</sup> Favour, C. B., *Proc. Soc. Exp. Biol. and Med.*, 1949, **70**, 369.

<sup>21</sup> Favour, C. B., Fremont-Smith, P., and Miller, J. M., *Am. Rev. Tub.*, 1949, **60**, 212.

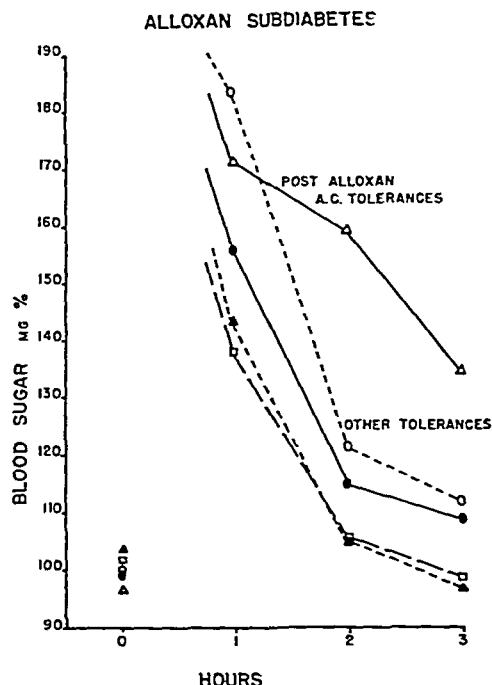


FIG. 1.  
Glucose tolerance curves before and after alloxan.

- Initial glucose tolerances
- Pre-alloxan A.C. tolerances
- ▲---▲ Post-alloxan glucose tolerances
- △---△ Post-alloxan A.C. tolerances
- Final glucose tolerances

Each point represents a mean blood sugar value as described in text.

erance tests the earliest figures were used. The control values for Rabbit 2 were not used because of the difference in method (see Table I), and none of the values for Rabbit 8 were used because of its spontaneous diabetes.

Two hours after the injection of glucose the mean blood sugar of the post-alloxan A.C. tolerance tests is 158.8 mg%, this is significantly higher than any of the other blood sugar means at that hour ( $p < 0.01$  for all the differences except that between the pre- and post-alloxan A. C. tolerance means, where  $p = 0.05$ ). At three hours the post-alloxan A.C. tolerance mean is 37.2 mg % higher than that of the post-alloxan glucose tolerances; this difference is of moderate significance ( $p < 0.05$ ). No other  $p$  values of 0.05 or less were found. Glucose tolerance curves for the five experimental situations have been

plotted in Fig. 1, using the same blood sugar means as in the statistical treatment.

Of the 6 rabbits which received alloxan, 4 were retested after a month or more, while one animal (No. 3) died 48 hours after a third dose of alloxan of 25 mg/k, and another (No. 7) died on 5/5 following a month of polydipsia and marked polyuria. Data on the retested animals is presented in Table II. Animal No. 4 subsequently died (4/26) following about 8 weeks of polydipsia and polyuria; it had not been retested since 1/13. Rabbit 5, which developed a diabetic ordinary glucose tolerance, was found to have hind limb paralysis shortly after its last test. Diarrhea supervened and the animal died immediately following the intraperitoneal injection of 60 cc of physiologic saline on the fifth day after this last tolerance test. At autopsy the bladder was found to be distended by about 100 cc of slightly turbid, albumin-free urine containing 179 mg% of sugar. The abdominal aorta exhibited two 0.5 cm long fusiform swellings; the intima in these areas was rough, friable, and pearly with loss of elasticity of the wall. The other organs appeared grossly normal. Histologic sections of the aorta showed medial sclerosis and calcification without significant intimal involvement. The aortas of Rabbits 4 and 7 appeared grossly normal.

**Discussion. Intravenous Glucose Tolerance.** In order to stabilize test conditions the rabbits were fasted<sup>9</sup> after a period of adequate carbohydrate feeding.<sup>10</sup> A uniform intravenous dose of 3 g of glucose (0.9-1.2 g/kg) was selected for the experiments in order to circumvent variability both in the time and in the degree of absorption of the sugar. Others have found intravenous tolerance methods satisfactory in normal rabbits,<sup>2,11-13</sup> and modification of moderate glucose dosage in

<sup>9</sup> Scott, E. L., *Arch. Int. Med.*, 1929, **43**, 393.

<sup>10</sup> Martin, E., Haworth, W. N., and Fantus, B., *Dextrose Therapy in Everyday Practice*, New York, 1937.

<sup>11</sup> Orr-Ewing, J., *J. Physiol.*, 1931, **73**, 365.

<sup>12</sup> Bang, I., *Der Blutzucker*, Wiesbaden, 1913.

<sup>13</sup> Oelkers, H. A., and Schutze, G., *Klin. Wchnschr.*, 1938, **17**, 871.



TABLE I.

Animal and wt	Date	Alloxan, mg/kg	Date	Glucose tolerance. Blood glucose level in mg/100 cc				Date	Adrenal cortex-glucose tolerance. Blood glucose level in mg/100 cc Hour			
				0	1	2	3		0	1	2	3
No. 1 2.9 kg		None	9/9	99	177	101	93	9/23	139	138	115	106
No. 2 2.9 kg			8/26*	111	276	188	—					
	8/30 10/22	23 29.3	10/28 11/11	101 97	110 111	97 106	95 97	11/4	77	159	215	206
No. 3 3.0 kg			9/23 9/28	108 113	104 —	125 102	97 117	9/30	92	166	141	127
	9/24	22										
No. 4 2.6 kg			10/7	86	199	139	146					
	10/8 10/15	24.2 15	10/26	122	120	110	99	10/28	104	177	139	124
No. 5 2.5 kg			10/7	104	154	124	115	10/14	72	173	120	104
	10/15 10/28	15 38	11/11 11/18	84 113	208 179	117 120	79 88†	11/15	75	213	159	104†
No. 6 3.6 kg			11/18	106	154	99	136	11/25	90	238	102	106
	12/7	41	12/16 12/23	102 102	127 111	101 95	93 90	12/19	119	177	145	117
No. 7 3.0 kg			11/25	95	146	101	65	12/2	—	184	148	131
	12/7 12/17	25 20	12/30 1/18	101 95	150 150	102 102	99 119	1/4	113	132	154	127
No. 8 2.9 kg		None	9/28	97	170	186	132	9/30	127	241	226	204

\* 7.4 g of 10% glucose injected intraperitoneally.

† Blood specimens drawn 15 minutes after the indicated hour.

cated. The initial and subsequent alloxan doses listed in Table I are approximate since in some instances there were small or moderate amounts of leakage at the hub of the needle, or of subcutaneous infiltration.

Autopsy was performed on rabbit No. 5 within 15 minutes of its death, and on rabbits Nos. 4 and 7 after approximately 8 hours. Pancreas, aorta and kidneys were studied, fixed in formalin, and stained by conventional histologic methods.

**Results.** Eight rabbits were studied, of which 6 received alloxan on one or two occasions. Blood sugar observations on these animals, before and after alloxan treatment, are presented in Table I. The data in Table I were treated statistically. Five means were computed for each hour of the glucose toler-

ance tests, using the following series: (1) The initial glucose tolerance tests; (2) the initial A.C. tolerance tests; (3) the glucose tolerance tests following the first effective dose of alloxan; (4) the A.C. tolerance tests immediately following the preceding group; and (5) the ordinary glucose tolerance tests following shortly after the fourth group. Calculations of the standard errors of the differences between each of these 5 blood sugar means were made for each hour (0, 1, 2, and 3) with the usual equation for small series, and *p* was determined, in each instance, from Fisher's *t* table. Where two successive tolerance tests of the same type were performed the later figures were used in computing the means, and only these figures appear in Table I; for the initial glucose tol-

paired carbohydrate metabolism not demonstrated by usual methods. As would be expected, a rabbit with diabetic glucose tolerance (No. 8) showed further impairment of its tolerance when pretreated with A.C.

*Alloxan Subdiabetes.* In these experiments one or two small doses of alloxan have produced a subdiabetic state in rabbits: namely disturbances in sugar metabolism which are so slight that they are not associated with either hyperglycemia or abnormal glucose tolerance. These slight physiologic disturbances have been demonstrated, in every instance, by the use of the A. C. tolerance test. This state is not discussed in the experimental literature, but it was probably produced by Shipley and Rannefeld.<sup>1</sup> These observers noted progressive and persistent impairment of glucose tolerance in rats injected with repeated 25 mg/kg doses of alloxan. In four of their rats, which had normal fasting blood sugars at the end of their alloxan courses, anterior pituitary extract produced a transient fasting hyperglycemia.

Three reports concerned with pancreatic islets suggest the presence of subdiabetes. These changes were reported in nondiabetic alloxan treated rats,<sup>22</sup> in 95% pancreatectomized rats prior to the onset of either latent or manifest diabetes,<sup>3</sup> and in one nondiabetic rabbit which received two 50 mg/kg doses of alloxan.<sup>23</sup> In each report the histologic findings were similar, namely hypertrophy and hyperplasia of the  $\beta$  cells in the larger islets, succeeded by  $\beta$  cell degranulation and degeneration, as few as 1/5 of the islets could be

involved. It seems not unlikely that comparable changes occurred in the present experiments.

The course of subdiabetes in these rabbits remains under observation. Progression has been observed in one animal, and maintenance of the subdiabetes for as long as 15 weeks has been the rule in the others. It would not be surprising if remission ultimately occurred in some animals, since spontaneous remission of manifest alloxan diabetes has been reported.<sup>24,25</sup> None of the morbid anatomical changes seen in human diabetes mellitus were found in the three animals thus far autopsied.

*Summary and conclusions.* 1. Small doses of adrenal cortex extract (A.C.) do not modify the glucose tolerance of fasted normal rabbits.

2. One or two small doses of alloxan (less than 40 mg/kg) do not produce more than transient changes in the blood sugar level or glucose tolerance of normal rabbits.

3. Six rabbits, so treated, showed a significant impairment of glucose tolerance when pretreated with A.C. (A.C. tolerance test); the hormone effect was transient.

4. By definition the treated rabbits had alloxan subdiabetes: they had impaired carbohydrate metabolism unaccompanied by fasting hyperglycemia, glycosuria, or impaired ordinary glucose tolerance.

5. Alloxan subdiabetes persisted in the rabbits for the duration of the experiment (up to 15 weeks).

6. A hormone sensitized glucose tolerance test, such as the A.C. tolerance test, should prove useful in studying problems in human subdiabetes and prediabetes.

<sup>22</sup> Hughes, H., and Hughes, G. E., *Brit. J. Exp. Path.*, 1944, **25**, 126.

<sup>23</sup> Dunn, J. S., Duffy, E., Gilmour, M. K., Kirkpatrick, J., and McLetchie, N. G. B., *J. Physiol.*, 1944, **103**, 233.

<sup>24</sup> Duffy, E., *J. Path. and Bact.*, 1945, **57**, 199.

<sup>25</sup> Lukens, F. D. W., *Physiol. Rev.*, 1948, **28**, 304.

TABLE II.  
Course of Subdiabetic Rabbits.

Animal	Date of last alloxan	Test date	Glucose tolerance test type	Blood sugar level in mg/100 cc Hr			
				0	1	2	3
No. 2	10/22	1/6	Ordinary	84	119	104	99
		11	A.C.	113	122	120	111
No. 4	10/15	11	Ordinary	88	136	115	102
		13	A.C.	125	221	186	184
No. 5	10/28	6	Ordinary	—	276	247	211
No. 6	12/7	13	Ordinary	88	99	92	99
		18	A.C.	90	164	129	104

proportion to body weight seems unnecessary.<sup>14</sup>

As expected<sup>12,13</sup> the one hour blood sugar levels, while high, showed wide variation. By 2 hours, however, the means of the ordinary glucose tolerance tests had returned to the levels of the fasting means. Sole exception was the mean of the initial tolerance tests, where the 2 hour mean was slightly (15.2 mg%), but not significantly, elevated. This variation is due principally to Rabbits 4 and 8 which later exhibited normal glucose tolerance curves; excitement may account for the form of their initial curves.<sup>15</sup> The return to normal by 2 hours agrees with the previous observations.<sup>2,11-13</sup>

At 3 hours the means of all of the ordinary glucose tolerance tests approximated the fasting means. In occasional rabbits there was a marked rebound from the 2 hour level, amounting to as much as 40 mg%.

*Modification of Glucose Tolerance by A.C.* The effects of adrenal corticoids<sup>16</sup> and of pituitary adrenocorticotrophic hormone (A.C.T.H.)<sup>17,18</sup> on carbohydrate metabolism are well known. Adrenal cortex extract was

chosen for the present experiments, rather than A.C.T.H., merely because it was immediately available. In selecting the dosage the aim was to use a quantity of hormone large enough to modify carbohydrate metabolism, yet small enough so that the glucose tolerance of a normal rabbit would be unaltered. The dose used,  $\frac{1}{2}$  cc/100 g body weight, is about 15% of the amount of A. C. which produces hyperglycemia in rats.<sup>19</sup> The preparation used was assayed by the manufacturer as containing "not less than 2.5 rat units/cc", and presumably it contained not much more. From Kuizenga's data<sup>20</sup> it may be calculated that the dosage of 11-dehydro-17-hydroxycorticosterone was approximately 0.04 mg/100 g body weight; this is about  $2\frac{1}{2}\%$  of the dose of corticoid which produces frank hyperglycemia in normal rats.<sup>21</sup>

The choice of dosage proved fortunate, since it did not modify the mean blood sugar levels of 4 rabbits subjected to A.C. tolerance tests prior to the administration of alloxan, while there was significant elevation of the mean 2 hour blood sugar level of the 6 alloxan treated rabbits subjected to A.C. tolerance tests. Since these alloxan treated rabbits had normal ordinary glucose tolerances, the A.C. tolerance test was effective in disclosing im-

<sup>14</sup> Lozner, E. L., Winkler, A. W., Taylor, F. H. L., and Peters, J. P., *J. Clin. Invest.*, 1941, **20**, 507.

<sup>15</sup> Himsworth, H. P., *J. Physiol.*, 1934, **81**, 29.

<sup>16</sup> Long, C. N. H., in Duncan, G. G., *Diseases of Metabolism*, 2nd ed., Philadelphia, Pa., 1947.

<sup>17</sup> Ingle, D. J., Li, C. H., and Evans, H. M., *Endocrinology*, 1946, **39**, 32.

<sup>18</sup> Conn, J. W., Louis, L. H., and Wheeler, C. E., *J. Lab. and Clin. Med.*, 1948, **33**, 651.

<sup>19</sup> Long, C. N. H., Katzin, B., and Fry, E. G., *Endocrinology*, 1940, **26**, 309.

<sup>20</sup> Kuizenga, M. H., in Ingle, D. J., *The Chemistry and Physiology of Hormones*, A.A.A.S. monograph 21a, 1943.

<sup>21</sup> Ingle, D. J., *Endocrinology*, 1941, **29**, 649.

TABLE I.  
Typical Systems.

Size of reservoir tubing	Delivery pressure, mmHg	Length of reservoir tubing, cm	Size of polythene tubing of delivery unit		
			Large (needle No. 18)	Medium (needle No. 18)	Small (needle No. 23)
			Delivery rate, cc per min.		
Infusion	155	100	20	4.5	.8
Aged 1 wk.		50	22	11	1.2
Single 3/8"	16	100	5	.8	.1
Aged 1 wk.		50	8	2.1	.2
Single 5/8"	14	100	3.5	.3	.08
Aged 1 wk.		50	5	1	.1
Double 5/8"	27	100	7	1.2	.15
Aged 1 wk.		50	10	2.1	.3
Double 1"	49	100	11	3	.4
Aged 1 wk.		50	18	5	.9

"Infusion" tubing, Products, Inc., Cuyahoga Falls, Ohio.

All other rubber tubing from Davol Rubber Co., Providence 2, R. I. They have received 1 1/2" tubing which has a delivery pressure of 41 mm single thickness and 70 mm double thickness, with comparable intermediate delivery rates.

Polythene tubing was supplied by Suprenant Electrical Insulation Company.

Large — 0.30 cm inside diam., 0.48 outside diam.

Medium—0.23 " " " 0.38 " "

Small — 0.11 " " " 0.24 " "

filled for delivery. The latex container is gradually distended with fluid, as the vacuum is created in the glass container, until it completely fills the bottle. The connection leading to the latex bag is then clamped off, and the apparatus is ready for delivery.

**Delivery.** A delivery unit of a suitable length and size of polythene tubing is connected to the clamped off tube connected to the latex bag. The needle or cannula of the delivery unit is inserted into the blood vessel or space desired and the clamp removed.

**Delivery Rate. Reproducibility.** The delivery rate of most of the fluid (60 to 80%) is linear, and for any given unit is constant within reasonable limits ( $\pm 3\%$ ) on successive runs with the same resistance and with the same solution being delivered. Similar apparatus made of similar materials will reproduce rates within 6%. Ageing of the tubing in contact up to 2 weeks with the solution to be delivered produces no significant change in delivery rate with such inert solutions as physiological saline and low concentrations of organic compounds of intermediate acidity and basicity. Treatment with depyrogenizing agents such as weak

bases (bicarbonate, sodium nitrite, green soap) had no effect on the delivery rate over 12 hours, but autoclaving drastically decreased the elasticity of the latex and affected the delivery rate. Sterilization for 12 hours with organic bactericides (Zephiran, Metaphen), however, had no noticeable effect.

**Applications.** This type of delivery unit is adaptable to many uses within a wide range of delivery rates (Table I). The desired delivery pressure may be roughly approximated by the selection of the type of tubing used in constructing the delivery unit. By using one latex container within another the arithmetic sum of their delivery pressures can be utilized. Delivery rate can be controlled most simply by increasing or decreasing the length of plastic tubing used for delivery. The rate may be slowed by increasing the viscosity of the solution, or by limiting the size of the air intake. If a relatively high viscosity fluid be admitted instead of air, the rate of delivery will be regulated by the rate of flow of the high viscosity fluid. To increase the rate of delivery, the pressure on the intake side may be increased by connecting two units in series. The latex containers

# 17269. A Self-Contained Method for the Administration of Fluids at Regular Rates.

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The continuous administration of fluids has been of considerable importance in the study of many phases of physiological phenomena.

Certain critical limits to any system for such administration must be adhered to: (1) the rate of delivery must be linear over the range demanded by the experimental procedure; (2) the system must be reasonably foolproof and simple to adjust; and (3) the system should be compact.

For some experimental purposes a rate of flow, constant within 0.1% is necessary, and methods have been designed to meet such tolerances.<sup>1,2</sup> Such systems are susceptible to mechanical failure, entail considerable supervision, and though modified<sup>3,4</sup> to allow movement of the subject, necessitate a continuous connection between the subject and some stationary structure, not always easy to maintain for long periods in the experimental animal.

For purposes less rigorous in requirements of tolerance of rate of flow, a method has been devised with possibilities of adaptation to a multiplicity of experimental uses, allowing complete freedom of movement to the recipient of the fluid.

**Construction.** A glass container of suitable volume is fitted with a 2 hole rubber stopper carrying 2 short lengths of glass tubing, one of which is flanged at the lower end. Over this flanged end which lies within the container, is fitted a short length of latex rubber tubing fastened securely with silk thread. The free end of this latex tubing is also tied

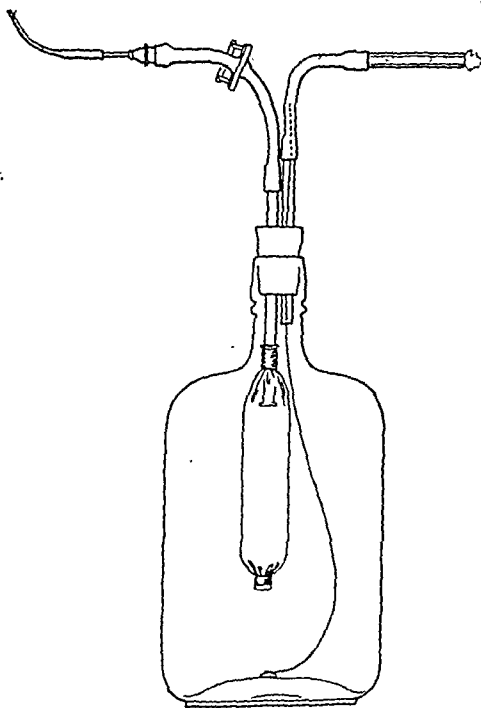


FIG. 1.

A typical delivery system shows the space relationships and mode of construction. The component parts are described in the text.

securely. To the second length of glass tubing is affixed a fine wire long enough to reach to the bottom of the glass container when the stopper is affixed (Fig. 1). The wire prevents tight sealing of the inflated tube to the glass wall.

**Operation. Filling.** Two short lengths of rubber tubing are attached to the outer ends of the glass tubing perforating the stopper, and the stopper is fitted tightly into the neck of the glass container. Vacuum is then applied to the tube to which the wire has been attached, while the end of the tubing carrying the latex container is connected to a reservoir of fluid with which the apparatus is to be

<sup>1</sup> Burn, J. H., and Dale, H. H., *J. Physiol.*, 1924, 59, 164.

<sup>2</sup> DeBakey, M. E., *New Orleans M. and S. J.*, 1934, 87, 386.

<sup>3</sup> Jacobs, H. D. R., *J. Lab. and Clin. Med.*, 1931, 16, 901.

<sup>4</sup> Stengel, A., Jr., and Vars, H. M., *J. Lab. and Clin. Med.*, 1939, 24, 525.

and easily managed method for cross transfusion.

The device consists of 4 units. 1. Suitable double lumen intravenous plastic cannulae. 2. A motor driven blood pump. 3. Bubble traps. 4. Metering and equalizing unit.

1. *The intravenous catheter.* Blood is withdrawn from and administered to the patient and donor through specially made intravenous catheters. These consist of extruded plastic double lumen tubes of an oval outer shape with an inside diameter of 2.5 mm for each conduit. The outside diameters of the catheters are 2.8 x 4 mm. The tip of the catheter is such as to provide for an outflow end protruding about 2" from the intake. The tip of the catheter is equipped with a thin finger-like projection which serves as a guide, the end of which terminates in a round, plastic-coated lead shot. The intake opening is fashioned with an overhanging lip which tends to prevent occlusion of the intake opening by the walls of the veins, by valves or by clots. The catheter is inserted into a vein and is pushed cardiad until the tip lies in the superior or inferior vena cava. The intake opening is upstream from the output opening and therefore no blood which is discharged through the output opening is aspirated through the intake opening when the rate of flow in the vein exceeds the rate of intake into the catheter. In our experience, this catheter has performed well. It carried an adequate volume of blood. Clotting is prevented by the intravenous injection of 1-2 mg heparin per pound of body weight and the catheter may be left *in situ*, without excessive reactions, for days at a time.

2. *The pump* (Fig. 1) propels blood by applying negative pressure to the catheter intake channel and imparting a positive pressure to the withdrawn blood. A simple commercially available pure gum rubber tube (1) is laid in a receiving channel and is then raised to connect with the outside valves (2,4) and compression unit (3). The valves and compression unit are actuated by a motor driven shaft (5) which is ground to provide cams (6) and eccentrics (7) which operate the valves and the compression unit respectively. In this manner when the "intake"

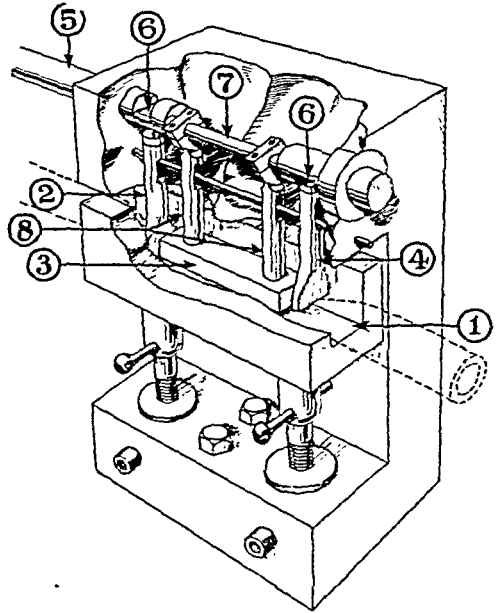


FIG. 1.  
Blood pump.

valve (2) is "down" the "outlet" valve (4) "up" and the compression bar (3) comes down, the tube will be compressed and blood will be forced forward. When the "outlet" valve is "down" the "intake" valve "up" and the compression bar is raised, blood is aspirated into the tube. The tube is occluded only at the relatively short segments where it is compressed by the valves; the excursion of the compression unit is adjusted to compress the tube, but not to squeeze it. This precaution has eliminated hemolysis. The pump requires a minimum of attention, such as an occasional lubrication of the moving parts with glycerin. It is possible to change the stroke volume by using tubes of different sizes and by raising or lowering the receiving channel. The minute volume can also be controlled by adjusting the speed of the drive shaft and can be adjusted to between 10 and 1000 cc per minute.

The pump used here consists of 2 identical devices, mounted in opposite directions, of equal dimensions, and adjustment which provide an excellent means of effecting cross transfusion of equal amounts of blood. Small gas bubbles may form occasionally in the

need not be similar in volume, expansion, or degree of elasticity. With solutions which react strongly with rubber, such a series system can be applied to deliver fluid at a given rate from an inelastic reservoir made of cellophane or viscose tubing.

Variations in pressure on the intake side of the apparatus can be made to regulate delivery to any given percentage by proper selection of the total pressure within the system. With thin walled tubing, variations of pressure up to 50% are in the physiological range. Thus, where the total delivery pressure is 50 mm Hg, variation of 10.0 mm in the circulatory system would cause a change of 20% in the rate of delivery. If however, the delivery pressure is 1200 mm, the variation in rate due to this will be less than 1%.

The apparatus is superior to gravity drip

in several respects. It can be placed in a light canvas harness on an experimental animal to deliver fluids over a long period during which the animal is at complete liberty. Delivery rates are variable over a range from 20 cc per minute to 0.08 cc per minute with only a minor change in the rubber stock used for the inside container.

*Summary.* 1. Various combinations of self-contained delivery units for the administration of fluid at regular rates are described, and the limits of usefulness of these units are delineated.

2. Combinations of such units to permit the administration of various types of fluids, including those likely to destroy the elasticity of rubber, are described.

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## 17270. Apparatus for Cross Transfusion.

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Cross transfusion, the exchange of blood between a donor and a patient, which results in continuous mixing of the blood of two bodies, may be used in conditions such as uremia, resulting from acute renal insufficiency from a variety of causes, and in the treatment of other non-transmissible diseases.

The ideal requirements of a method for successful cross transfusion are: 1. Exchange of blood at a rate which may be varied from 0.5 to 10 liters per hour. 2. No chemical changes or hemolysis occurring in the transmitted blood. 3. Accurate measurement of the transferred blood. 4. Automatic equalization of the amounts of blood transferred in both directions. 5. The use of venous blood only, because donors and patients cannot be expected to sacrifice arteries every time that this procedure is applied.

In the past, cross transfusion has been

applied in a variety of ways, none of which has been completely satisfactory. The method of paired arterial-venous anastomosis does not permit measurement of the exchanged blood and is obviously unsuitable. The multiple syringe method and the bottle method are tedious, slow and prone to introduce airborne infections. Pumps which operate on the "leaky valve" principle exhibit fluctuations in output which are proportional to the resistance encountered by the outflow. The worm pump (Pennell) and the roller pump (DeBakey) methods hemolyze at least 0.1% of the transferred blood and do not measure the transferred blood with sufficient accuracy. Although 0.1% of hemolysis is a tolerably small amount for ordinary direct transfusions of about 500 cc, yet, when quantities up to 50,000 cc are involved, the amount of hemolysis caused by such pumps may prove injurious.

This report deals with a new device which satisfies the above criteria for a safe, efficient

\* Assisted by Dr. Joseph Miller. Supported by a grant from Mr. Emil Friedlander.

flows into the measuring chambers. Blood from animal X is now pumped into chamber B1 instead of Chamber A1, thereby expelling into animal X the blood from animal Y which had been collected in chamber B2 during the preceding cycle. The number of changes of the valve mechanism is recorded automatically on a meter and provides an easy and convenient way of determining mean blood flow and total amount exchanged. The volume of the chambers is 50 cc each.

This arrangement has the following advantages: It measures and equalizes the flow of blood in both directions automatically. It provides for simultaneous withdrawal and administration of equal amounts of blood. The accuracy of the registration of blood flow is about  $\pm 1-3$  cc in 10,000 cc, depending upon the fit of the piston and on the pres-

sure differential in the two circuits. The device has been found completely satisfactory in experiments involving the exchange of a total, to date, of 500,000 cc of blood in both animals and human beings. The amount of heparin needed to prevent coagulation is within the clinically acceptable limits. Hemolysis does not occur if the pump is properly adjusted. Cross transfusion itself has proved to be a safe, efficient and relatively easy procedure, when performed with the apparatus described above. No fatalities attributable to the procedure itself have occurred.

*Summary.* A safe, effective and easily controlled apparatus for cross transfusion is described.

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### 17271. Vasomotor Reactions in the Mesenteric and Serosal Capillary Bed During Fright and Violent Muscular Activity.\*

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Recently, technics were devised for painless exposure, with local anesthesia, of the guinea pig mesentery and gut and its maintenance in a warmed chamber where the capillary bed could be observed continuously with the microscope.<sup>1</sup> This permitted study of peripheral vasomotor reactions, and vascular responses to drugs without possible variables introduced by general anesthesia. In addition, the fact that the animal studied was fully conscious afforded the opportunity to witness the behavior of terminal arterioles and capillaries in this splanchnic area during

various emotional states and spontaneous muscular activity.

*Methods.* Studies were made on 20 animals receiving a normal well supplemented diet, and on 21 animals in moderately advanced Vitamin C deficiency after 3-4 weeks on a standard scorbutogenic diet. The apparatus and diet have been described previously.<sup>1</sup> In brief, it includes holding a trained animal under light restraint, with cautious painless exposure of the mesentery and gut via a denervated or procaine field block in the flank. The preparation is maintained in a suitable warm chamber and continuously bathed with a drip solution of warmed buffered Ringer-gelatin solution, while being observed under the microscope.

*Results.* A. Animals receiving a "normal" diet. The changes found in the capillary bed of this splanchnic region in the control animals could be divided into 2 major categories. The

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\* This study was supported in part by a grant in aid from the Nutrition Foundation to Syracuse University College of Medicine.

† Present address: Department of Medicine, Cornell University Medical College, New York Hospital, New York.

<sup>1</sup> Lee, Richard E., and Lee, Nina Z., *Am. J. Physiol.*, 1947, 149, 465.



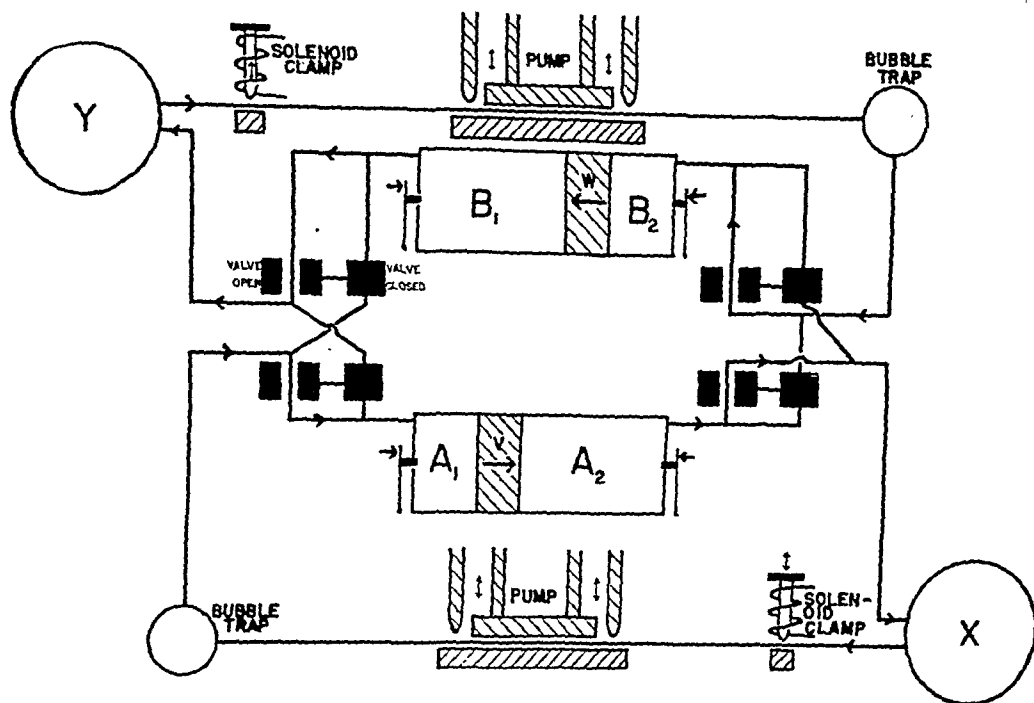


Fig. 2.  
Diagram of blood flow in the cross transfusion apparatus.

blood because of the negative pressure exerted by the pump when it is withdrawing blood from the vein. Also, variations in stroke volume of the pump may occur because of changes in pressure in the intake channel, which are possibly caused by a reduction of the effective size of the intake opening. These two sources of error are corrected by bubble traps and a measuring and equalizing unit described below.

3. *Bubble traps.* These consist of 2 upright, cylindrical plastic chambers, one inch in diameter and  $3\frac{1}{2}$ " in height. Blood flows into one end of a chamber, near the top, and flows out at the bottom, allowing any air bubbles contained in it to collect at the top. Near the top of the cylinder there is a flexible plastic membrane which forms the floor of another plastic chamber which is filled with air and transmits variations in pressure to a U-type mercury manometer. Just underneath the plastic membrane there is a vent-like opening, to allow for adjustment of the blood level within the bubble trap and for

the introduction of liquids into the blood-stream.

This device removes all but the very smallest air bubbles, allows for the constant indication of pressure and the administration of anticoagulants, drugs, etc., to either blood-stream during the experiment.

4. *The meter.* (Fig. 2) The metering unit receives blood into cylindrical chambers A1 and B2 from bodies of the subjects X and Y. As the blood is pumped into chambers A1 and B2, two floating plastic pistons, V and W, move toward the opposite ends of the chambers and expel the contents of chambers A2 and B1 into bodies X and Y respectively. At the end of the excursion of the floating pistons a micro-switch mechanism provides for the activation of solenoid clamps which stop the flow of blood to whichever chamber has been filled. When both chambers are filled, a valve mechanism is moved by means of a motor. As soon as the movement of the valve-changing mechanism is completed the solenoid clamps are released and blood again

pheral vascular system in this nutritional deficiency, and its possible significance has been reported previously.<sup>1</sup>

*Discussion.* Although the mucosal blood vessels were not observed directly in this study, the complete cessation of blood flow found frequently in the small arteries and arterioles supplying the mesentery and traversing it to supply the intestine support the assumption that accompanying the "flight reaction" there is often a complete stagnation of blood flow in this viscus. This is apparently maintained throughout the period of response to the stimulus, and subsequently returns slowly. Of particular interest is the fact that even at the height of response, continuous blood flow through the arterial arcades and the larger venules and veins in the mesentery affords a means of draining much of the residual capillary blood in this region and returning it, along with the blood shunted to the arcade system, back to the general circulation. One can assume that this would considerably increase the volume of blood available for other somatic areas during the period of active vascular response.

The absence of vasospasm in the mesentery and serosa during the flight response in scor-

butic animals is associated with a general depression of arteriolar and precapillary tone, and impaired venular flow.<sup>1</sup> It remains to be determined whether these manifestations of ascorbic acid deficiency are a result of general tissue disfunction or are perhaps a specific defect in some vasotonic mechanism.

*Summary and conclusions.* 1. Technics were devised for viewing the mesenteric and serosal capillary bed of guinea pigs not under a general anesthetic agent.

2. The degree of emotional response to auditory stimuli could be correlated with the magnitude of changes in the visualized splanchnic vessels.

3. (a) A "startle response" was occasionally accompanied by transient slight vasoconstriction and slowing of blood flow.

(b) A "flight response" was frequently characterized by intense vasoconstriction of arterioles, complete stagnation of capillary blood flow, and a drainage of residual capillary blood into patent venules.

3. Certain "key sites" along the arteriolar tree have a lower threshold of response than remaining areas, and exert much control of arteriolar flow by sphincter-like activity.

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## 17272. Reversal of Insulin-Induced Hypoglycemia in Chick Embryos by Nicotinamide and $\alpha$ -Ketoglutaric Acid.\*

EDGAR ZWILLING.

From Storrs Agricultural Experiment Station, University of Connecticut, Storrs, Conn.

In a series of papers Landauer and his collaborators<sup>1-4</sup> have described the teratogenic effects which insulin produces when it is in-

jected into the yolk sac of chick embryos. When the insulin is applied in this manner to early embryos (0-72 hrs) caudal defects (rumplessness) are the most frequently encountered anomalies. When injected into older embryos (96-168 hrs) the insulin causes a high incidence of disproportionate shortening of the legs (micromelia). These effects of insulin disappear when the insulin is inactivated and reappear when inactivated insulin is reactivated. Landauer<sup>5</sup> demonstrated that when

\* This investigation was aided, in part, by a grant from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council.

<sup>1</sup> Landauer, W., *J. Exp. Zool.*, 1945, **98**, 65.

<sup>2</sup> Landauer, W., and Lang, E. H., *J. Exp. Zool.*, 1946, **101**, 41.

<sup>3</sup> Landauer, W., and Bliss, C. I., *J. Exp. Zool.*, 1946, **102**, 1.

<sup>4</sup> Landauer, W., *J. Exp. Zool.*, 1947, **105**, 145.

<sup>5</sup> Landauer, W., *J. Exp. Zool.*, 1948, **109**, 283.

terms "startle reaction" and "flight reaction" are based on the degree of overt manifestation of "emotional" disturbances.

1. The "startle reaction." It was first noticed that following a sudden loud noise, the animal being examined might shudder, or otherwise appear startled. The great majority of such responses were not accompanied by any observed phenomena in the splanchnic vascular bed. In approximately 20% of such situations, however, there was a slight narrowing of mesenteric and serosal arterioles of diameters ranging from 50-80  $\mu$  with general slowing of capillary blood flow. Precapillary sphincters usually narrowed partially. This vasoconstriction generally began 1-2 seconds after the brief startle response by the animal, and lasted 5-10 seconds at most. It frequently involved isolated regions of the arteriole, producing a band-like constriction of 30-40  $\mu$  in length, in which the arteriolar diameter was reduced occasionally by 25-50%. As repeated "startle" responses were induced in the same animal, it was noted that these sphincterlike constrictions of the arterioles invariably occurred at the same locus. Apparently, certain "key sites" along the small peripheral vessels, perhaps associated with the terminals of particular nerve fibers, are capable of response at levels of psychic stimulation below those required for a more generalized vasoconstriction.

2. The "flight reaction." Occasionally, the auditory and other stimuli would be followed by a much more vigorous and prolonged sudden reaction, in which the animal would struggle violently to escape from his bonds. This muscular effort (and apparently emotional disturbance) usually lasted for 5-20 seconds, then ceased as suddenly as it had begun. Motion of the fields under observation often prevented accurate visualization during these episodes.

Of those instances in which the mesentery remained relatively undisturbed, approximately 25% were accompanied by no observed vascular changes. In 50% of the episodes, vascular phenomena were found resembling those of the startle reaction but generally of greater magnitude. The remaining 25% of the "flight reactions" were ac-

companied by profound changes in the vessels. Within 1-2 seconds after the onset of struggling the "key sites" of the larger arterioles became greatly narrowed to completely occluded. Apparently at the same time, narrowing occurred in the entire remainder of the arteriolar components, with marked to complete constriction of all precapillary sphincters in the mesentery and serosa. This intense and widespread vasoconstriction resulted in a complete cessation of observed arteriolar blood flow to the gut, with limitation of active circulation to the larger mesenteric arterial arcades. The blood flow in the venules and veins became very sluggish, but continued. This afforded a relatively rapid drainage into the general circulation of much of the residual capillary blood.

This picture of complete capillary stagnation lasted throughout the period of excitement and muscular activity. When struggling ceased, frequently no change was seen for 5-10 seconds. The first areas to release their spasm were the "key sites" along the larger arterioles. Flow gradually increased through the narrowed arteriolar tree along the more direct A-V channels. Within 15-20 seconds, the smaller arterioles began to dilate, resulting in augmentation of flow in the arteriolar net. It was frequently 1-2 minutes, however, before sufficient pre-capillary sphincters had opened to permit a return of adequate capillary circulation. Following the disappearance of this vasospastic state, no further obvious changes were noted in this study.

In 3 instances, observations on arteriolar and precapillary sensitivity to topical epinephrine were made immediately after the resumption of a normal capillary flow. In each it was found to be at least 300% greater than control readings of 1:2,000,000. Five minutes later it had returned to normal values.

B. The scorbutic animals. Although the "startle responses" and the "flight responses" occurred as frequently and as readily in the scorbutic guinea pigs as compared to the pair-fed controls, circulatory changes during or following these episodes were not observed. The relatively depressed condition of the peri-

pheral vascular system in this nutritional deficiency, and its possible significance has been reported previously.<sup>1</sup>

*Discussion.* Although the mucosal blood vessels were not observed directly in this study, the complete cessation of blood flow found frequently in the small arteries and arterioles supplying the mesentery and traversing it to supply the intestine support the assumption that accompanying the "flight reaction" there is often a complete stagnation of blood flow in this viscus. This is apparently maintained throughout the period of response to the stimulus, and subsequently returns slowly. Of particular interest is the fact that even at the height of response, continuous blood flow through the arterial arcades and the larger venules and veins in the mesentery affords a means of draining much of the residual capillary blood in this region and returning it, along with the blood shunted to the arcade system, back to the general circulation. One can assume that this would considerably increase the volume of blood available for other somatic areas during the period of active vascular response.

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## 17272. Reversal of Insulin-Induced Hypoglycemia in Chick Embryos by Nicotinamide and $\alpha$ -Ketoglutaric Acid.\*

EDGAR ZWILLING.

From Storrs Agricultural Experiment Station, University of Connecticut, Storrs, Conn.

In a series of papers Landauer and his collaborators<sup>1-4</sup> have described the teratogenic effects which insulin produces when it is in-

jected into the yolk sac of chick embryos. When the insulin is applied in this manner to early embryos (0-72 hrs) caudal defects (rumplessness) are the most frequently encountered anomalies. When injected into older embryos (96-168 hrs) the insulin causes a high incidence of disproportionate shortening of the legs (micromelia). These effects of insulin disappear when the insulin is inactivated and reappear when inactivated insulin is reactivated. Landauer<sup>5</sup> demonstrated that when

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<sup>4</sup> Landauer, W., *J. Exp. Zool.*, 1947, **105**, 145.

<sup>5</sup> Landauer, W., *J. Exp. Zool.*, 1948, **109**, 283.

nicotinamide is injected into the yolk sac either shortly before, after or simultaneously with insulin the incidence of micromelia is reduced from 41 - 53% to 3 - 9% depending on individual experiments. When the same experiments were repeated on earlier stages it was found that nicotinamide also reduces the incidence of rumplessness. In like manner  $\alpha$ -ketoglutaric acid reduces the incidence of micromelia, though not as effectively as nicotinamide, but does not, however, exert much of an inhibiting effect on the rumpless-inducing properties of the insulin.

Zwilling<sup>6</sup> showed that the injection of insulin into the yolk sac of 120 hr embryos is followed by an hypoglycemia which, in some cases, persists for 8 days. The blood sugar in all embryos is restored to normal levels by the fourteenth day of development. In the course of this investigation it was found that a very good correlation exists between the degree and persistence of the hypoglycemia and the degree and incidence of micromelia. Factors which increase the micromelic effects (such as adrenal cortical extracts, Landauer<sup>7</sup>) also exaggerate the hypoglycemic effects.

It was of some interest to us to determine whether nicotinamide and  $\alpha$ -ketoglutaric acid have any effect on the insulin-induced hypoglycemia. We found that these substances do decrease the incidence of hypoglycemia and that this decrease follows the same order that it does for their micromelia-alleviating capacities; *i.e.* the effect is more pronounced with nicotinamide.

**Material and method.** Eggs from unselected White Leghorn hens were used in this work. They were all injected at 120 hours as described by Landauer.<sup>1</sup> When two substances were used they were injected separately, but in sufficiently rapid succession so that they may be considered simultaneous injections. Blood was obtained and assayed for reducing substances by the technic described previously.<sup>6</sup> For each stage blood samples were taken from embryos which had been injected with insulin alone (2 units), nicotina-

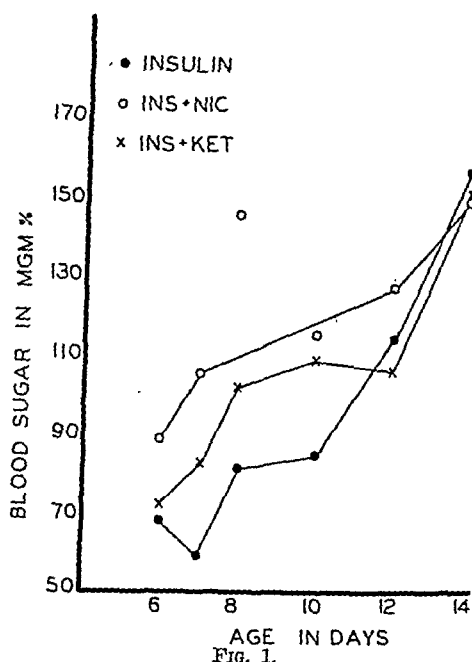


FIG. 1.  
Course of blood sugar in chick embryos treated with: insulin alone (2 units), insulin + nicotinamide (18.9 mg), and insulin +  $\alpha$ -ketoglutaric acid (20.2 mg) at 5 days. Each point represents the average of blood sugar values for all embryos assayed at a given stage.

mid alone (18.9 mg) or  $\alpha$ -ketoglutaric acid alone (20.2 mg) and both insulin and one of the other two substances. The sequence of obtaining blood from the embryos was altered sufficiently so that it could not influence the results.

**Results.** The data from these experiments are presented in Fig. 1 and in Table I. Since neither nicotinamide alone nor  $\alpha$ -ketoglutaric acid alone cause any marked deviation from normal blood sugar levels the data obtained for these substances have been omitted. All of the points on these curves represent averages for all blood sugar values obtained for a given stage. This explains the difference between the curve for 2 units of insulin presented here and that presented in the previous paper.<sup>6</sup> In the latter the points represented the average values for all hypoglycemic embryos at any given stage. However, these data for insulin alone duplicate those of last year in all essential respects. It can be seen that both nicotinamide and  $\alpha$ -ketoglutaric

<sup>6</sup> Zwilling, E., *J. Exp. Zool.*, 1948, 109, 197.

<sup>7</sup> Landauer, W., *Endocrinol.*, 1947, 41, 489.

TABLE I.  
Summary of Blood Sugar Data from Embryos Injected with Insulin (2 Units), Insulin + Nicotinamide (18.9 mg) and Insulin +  $\alpha$ -ketoglutaric Acid (20.2 mg). All injections were into the yolk sac of 5 day embryos. In the last column under each substance the range of blood sugar values is given for only the hypoglycemic embryos. Values in the normal range have been omitted.

Age in days	2 units insulin				Insulin + nicotinamide				Insulin + $\alpha$ -ketoglutaric acid			
	No. of embryos	No. hypoglycemic	Range of hypoglycemia, mg %	No. of embryos	No. hypoglycemic	Range of hypoglycemia, mg %	No. embryos	No. hypoglycemic	No. embryos	No. hypoglycemic	Range of hypoglycemia, mg %	No. embryos
6	13	13	43.4-97.2	10	9	62.5-108.6	10	9	10	9	42.7-83.9	
7	13	13	38.0-85.3	9	4	67.8-90.0	10	9	10	9	59.4-103.0	
8	16	15	49.8-101.2	10	2	100.5-105.6	18	11	18	11	61.3-105.0	
10	16	13	40.0-105.1	11	4	66.9-96.1	18	10	18	10	43.1-108.2	
12	13	6	34.7-95.8	9	3	42.4-94.0	8	4	8	4	32.1-88.2	
14	7	0		6	0		7	0	7	0		

acid reduce the average hypoglycemia and that this effect of the nicotinamide is evident within twenty-four hours of the time that it is administered. From the data presented in Table I it is evident that this is due, in large part, to a reduction in the number of embryos which are hypoglycemic at any one time. However there is also a decrease in the degree of hypoglycemia of the individual embryos. Nicotinamide is more effective than  $\alpha$ -ketoglutaric acid in producing both of these results. This correlates well with the fact that cases of extreme micromelia are not found following injection of insulin + nicotinamide, while they do occur after injection of insulin +  $\alpha$ -ketoglutaric acid. The one discordant point (insulin + nicotinamide at 8 days) may be due to a sampling error. However, there is a possibility that it may, in part, represent an actual increase. Not only were there fewer cases of hypoglycemia but the range of "normal" values was higher than usual. A similar phenomenon has been observed in our other work; it seems as though recovery to normal levels may be accompanied by an over-compensation.

*Discussion.* Again we have an instance in which the level of blood sugar varies as does the degree of micromelia. In this instance nicotinamide and  $\alpha$ -ketoglutaric acid are instrumental both in decreasing the incidence of micromelia and in restoring the blood sugar of the embryos to more normal levels. It might be pointed out that the curve for insulin + nicotinamide described here is very similar to the one which describes the course followed by the blood sugar in embryos which receive only insulin but which show none of the teratogenic effects of that substance.<sup>6</sup>

The causal relationship between the morphological effects of insulin and its hypoglycemic action in the chick embryo is still obscure. It is not yet certain that these effects are *directly* due to a decrease in available carbohydrate energy, though it is evident that the carbohydrate metabolism is involved. In like manner the mechanism of the alleviating action of the nicotinamide is not clear. It may either be a result of its restoring the blood sugar to normal levels or due to a more direct action on the embryonic tissues.

nicotinamide is injected into the yolk sac either shortly before, after or simultaneously with insulin the incidence of micromelia is reduced from 41 - 53% to 3 - 9% depending on individual experiments. When the same experiments were repeated on earlier stages it was found that nicotinamide also reduces the incidence of rumplessness. In like manner  $\alpha$ -ketoglutaric acid reduces the incidence of micromelia, though not as effectively as nicotinamide, but does not, however, exert much of an inhibiting effect on the rumpless-inducing properties of the insulin.

Zwilling<sup>6</sup> showed that the injection of insulin into the yolk sac of 120 hr embryos is followed by an hypoglycemia which, in some cases, persists for 8 days. The blood sugar in all embryos is restored to normal levels by the fourteenth day of development. In the course of this investigation it was found that a very good correlation exists between the degree and persistence of the hypoglycemia and the degree and incidence of micromelia. Factors which increase the micromelic effects (such as adrenal cortical extracts, Landauer<sup>7</sup>) also exaggerate the hypoglycemic effects.

It was of some interest to us to determine whether nicotinamide and  $\alpha$ -ketoglutaric acid have any effect on the insulin-induced hypoglycemia. We found that these substances do decrease the incidence of hypoglycemia and that this decrease follows the same order that it does for their micromelia-alleviating capacities; *i.e.* the effect is more pronounced with nicotinamide.

**Material and method.** Eggs from unselected White Leghorn hens were used in this work. They were all injected at 120 hours as described by Landauer.<sup>1</sup> When two substances were used they were injected separately, but in sufficiently rapid succession so that they may be considered simultaneous injections. Blood was obtained and assayed for reducing substances by the technic described previously.<sup>6</sup> For each stage blood samples were taken from embryos which had been injected with insulin alone (2 units), nicotina-

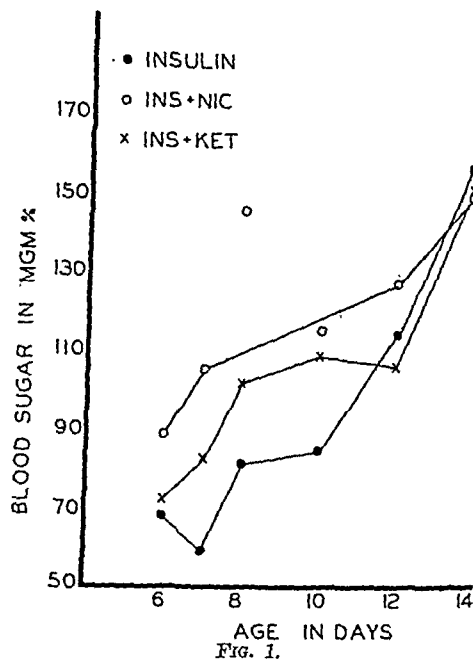


FIG. 1.  
Course of blood sugar in chick embryos treated with: insulin alone (2 units), insulin + nicotinamide (18.9 mg), and insulin +  $\alpha$ -ketoglutaric acid (20.2 mg) at 5 days. Each point represents the average of blood sugar values for all embryos assayed at a given stage.

mid alone (18.9 mg) or  $\alpha$ -ketoglutaric acid alone (20.2 mg) and both insulin and one of the other two substances. The sequence of obtaining blood from the embryos was altered sufficiently so that it could not influence the results.

**Results.** The data from these experiments are presented in Fig. 1 and in Table I. Since neither nicotinamide alone nor  $\alpha$ -ketoglutaric acid alone cause any marked deviation from normal blood sugar levels the data obtained for these substances have been omitted. All of the points on these curves represent averages for all blood sugar values obtained for a given stage. This explains the difference between the curve for 2 units of insulin presented here and that presented in the previous paper.<sup>6</sup> In the latter the points represented the average values for all hypoglycemic embryos at any given stage. However, these data for insulin alone duplicate those of last year in all essential respects. It can be seen that both nicotinamide and  $\alpha$ -ketoglutaric

<sup>6</sup> Zwilling, E., *J. Exp. Zool.*, 1948, 109, 197.

<sup>7</sup> Landauer, W., *Endocrinol.*, 1947, 41, 489.

them<sup>5</sup> has found the same apparent contradiction in rats fed anti-thyroid drugs. The animals in his experiments lost weight, probably due to a restriction in food intake, but the protein content of the liver was remarkably increased. Leatham and Seeley<sup>6</sup> have also demonstrated that the plasma proteins of rats fed the anti-thyroid drugs were increased with the largest increase occurring in plasma globulins.

In an attempt to resolve these apparent contradictions, the following experiments were designed to determine the nitrogen balance at various levels of methionine and thiouracil in the diet and to determine the nitrogen content of the plasma and tissues.

**Methods.** Nitrogen balance indexes<sup>†</sup> were determined on adult rats of the Long-Evans Strain using the diets and technics previously described for the dog.<sup>7</sup> The diet contained 12% casein on a dry weight basis and the amino acid and thiouracil were added at various levels. Rats were pair fed with those receiving methionine (4.8%) where the food intake was markedly restricted. Experiments were also performed on animals fed *ad libitum* as a secondary control. The experiments were continued for 20 days with urine and fecal collections throughout the period. At the end of the experiment the animals were bled by heart puncture and autopsied. The plasma was analyzed by the method of Howe<sup>8</sup> as modified by Robinson, Price, and Hogdem.<sup>9</sup> The livers and kidneys were dried to constant weight at 95°C and were then analyzed for nitrogen by the micro-Kjeldahl procedure.

<sup>5</sup> Leatham, J. H., *Proc. Soc. Exp. Biol. and Med.*, 1946, **61**, 203.

<sup>6</sup> Leatham, J. H., and Seeley, R. D., *Am. J. Physiology*, 1947, **149**, 361.

<sup>†</sup> The nitrogen balance index may be defined by the equation

$$NB = K(AN) - NE^0$$

Where NB is the nitrogen balance, AN the absorbed nitrogen, and  $NE^0$  is the excretion of nitrogen on a protein free diet. Under these conditions, K may be defined as the nitrogen balance index.

<sup>7</sup> Allison, J. B., and Anderson, J. A., *J. Nutrition*, 1945, **20**, 413.

<sup>8</sup> Howe, P. E., *J. Biol. Chem.*, 1921, **49**, 93.

<sup>9</sup> Robinson, H. W., Price, J. W., and Hogdem, C. G., *J. Biol. Chem.*, 1937, **120**, 491.

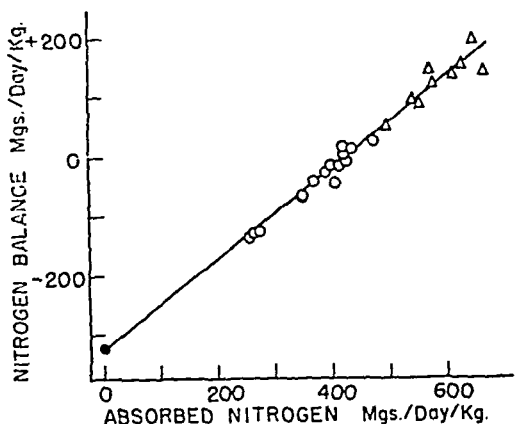


Fig. 1.

The relationship between nitrogen balance and absorbed nitrogen in rats fed a 12% casein ration *ad libitum* as indicated by the triangles, a 12% casein ration on a restricted food intake as indicated by the open circles, and a protein-free diet as indicated by the closed circle.

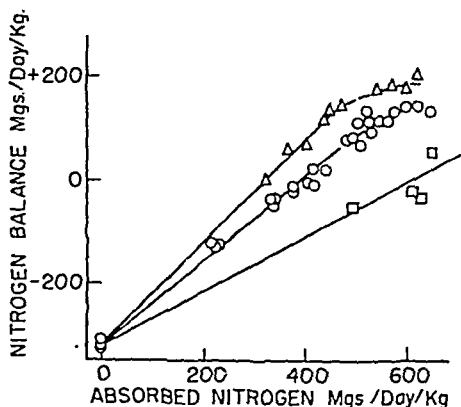


Fig. 2.

The relationship between nitrogen balance and absorbed nitrogen in rats fed a diet of 12% casein as indicated by the open circles, 12% casein to which was added 0.67% methionine ( $\Delta$ ), and 12% casein to which 0.6% thiouracil ( $\square$ ) had been added.

The urine and feces were also analyzed by the micro-Kjeldahl procedure. The Folin method<sup>10</sup> was used for the analysis of creatine and creatinine.

**Results.** The points plotted in Fig. 1 illustrate data obtained on rats fed a 12% casein ration (dry weight basis) *ad libitum* as shown by the triangles, and rats fed the 12% casein

<sup>10</sup> Folin, O. S., *J. Biol. Chem.*, 1914, **17**, 469.



We have found that sulfanilamide, which produces essentially the same micromelia as insulin,<sup>8</sup> does not alter the blood sugar (unpublished). However its micromelic effects are also reversed by nicotinamide (unpublished). There is a possibility that the low concentration of reducing sugars in the insulin-treated embryos may result in a higher nicotinamide requirement, while the sulfonamide may affect the nicotinamide content in another fashion. Levy and Young<sup>9</sup> have shown that virtually all of the nicotinamide (or nicotinic acid) in the chick embryo is bound in the diphosphopyridine nucleotide (coenzyme I). They also indicate (as has been found in other animal tissues<sup>10</sup>) that excess nicotinic acid (or amide) does not result in an increase in the coenzyme beyond its maximum levels. If these same conditions obtain under the abnormal circumstances induced by insulin (and sulfanilamide), then it seems likely that these substances may interfere, in some manner, with the synthesis of the coenzyme.

The hypoglycemia-alleviating effect of nicotinamide demonstrated here is not without

<sup>8</sup> Aneel, P., *Ann. d'Endocrinol.*, 1945, **6**, 1.

<sup>9</sup> Levy, M., and Young, N. F., *J. Biol. Chem.*, 1948, **176**, 185.

<sup>10</sup> Schlenk, F., A Symposium on Respiratory Enzymes, Univ. of Wis. Press, 1942.

precedent. Burke and McIntyre<sup>11</sup> have shown that this substance increases the tolerance of rats to insulin; a decrease in the duration of hypoglycemia was noted following the injection of a standard dose of insulin into nicotinamide-treated animals. These are essentially the same results which we have obtained with chick embryos.

That  $\alpha$ -ketoglutaric acid also prevents insulin abnormalities as well as decreasing the duration of the hypoglycemia may indicate that pyruvate metabolism is, in some way, inhibited (possibly via coenzyme lack) and that this alternate pathway may be utilized as an energy source. At any rate these data indicate that carbohydrate metabolism is of considerable importance for normal limb formation in chick embryos.

**Summary.** The duration of insulin-induced hypoglycemia is reduced following the administration of nicotinamide and  $\alpha$ -ketoglutaric acid to insulin treated chick embryos. Nicotinamide, which is the more effective in preventing insulin-induced abnormalities, is also more effective in decreasing the duration of the hypoglycemia.

<sup>11</sup> Burke, J. C., and McIntyre, A. R., *J. Pharm. and Exp. Therap.*, 1939, **67**, 142.

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## 17273. Influence of Methionine and Thiouracil on Nitrogen Balance Index and Organ Weights of Adult Rat.\*

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Allison, Anderson, and Seeley<sup>1</sup> have demonstrated that small quantities of methionine conserve body nitrogen when added to the diet of the dog. Earle, Small, and Victor<sup>2</sup> and Reisen, *et al.*<sup>3</sup> have found, however, that larger

amounts of the amino acid will cause a marked loss in body weight in rats. This is in apparent contradiction to Harrison and Long<sup>4</sup> who reported that a diet containing 2% of methionine would increase the liver nitrogen of rats. Lea-

\* Presented as partial fulfillment of the degree of Doctor of Philosophy at Rutgers University.

<sup>†</sup> Gerard Swope Fellow of the General Electric Company.

<sup>1</sup> Allison, J. B., Anderson, J. A., and Seeley, R. D., *J. Nutrition*, 1946, **33**, 361.

<sup>2</sup> Earle, D. J., Jr., Small, K., and Victor, J., *J. Exp. Med.*, 1942, **76**, 317.

<sup>3</sup> Reisen, W. H., Schweigert, B. S., and Elvehjem, C. A., *Arch. Biochem.*, 1946, **10**, 307.

<sup>4</sup> Harrison, H. C., and Long, C. N. H., *J. Biol. Chem.*, 1945, **161**, 547.

TABLE II.

Data Obtained on Autopsy of Rats Fed a 12% Casein Diet *ad libitum* (A) and a 12% Casein Diet on a Restricted Food Intake (R) to Which Was Added Varying Amounts of Methionine Thiouracil.

Dietary regime	Thyroid, mg/100 g., B.W.	Liver, mg/100 g., B.W.	Kidney, mg/100 g., B.W.
Control (R)	6.7 $\pm$ 0.9	3230 $\pm$ 48	684 $\pm$ 24
" (A)	6.2 $\pm$ 0.5	3352 $\pm$ 52	691 $\pm$ 36
Thiouracil			
.12 (R)	9.5 $\pm$ 0.8	3450 $\pm$ 142	673 $\pm$ 32
.67 (R)	12.8 $\pm$ 1.2	3892 $\pm$ 210	684 $\pm$ 37
.67 (A)	16.2 $\pm$ 1.3	4221 $\pm$ 178	684 $\pm$ 31
Methionine			
.67 (A)	6.8 $\pm$ 0.7	2931 $\pm$ 96	811 $\pm$ 82
.67 (R)	6.4 $\pm$ 0.6	3193 $\pm$ 103	705 $\pm$ 40
2.4 (R)	7.2 $\pm$ 0.9	2961 $\pm$ 80	850 $\pm$ 32
4.8 (R)	8.7 $\pm$ 0.9	3290 $\pm$ 92	925 $\pm$ 46

TABLE III.

Influence of Various Concentrations of Methionine and Thiouracil on Liver Nitrogen of Rats Receiving a 12% Casein Basal Ration.

Dietary regime, sulfur	No. of animals	Liver N, %	Total Liver N, g/100 g., B.W.	H <sub>2</sub> O, %
Casein (A)	18	9.2 $\pm$ .6	.098 $\pm$ .004	69.8 $\pm$ 1.2
" (R)	14	9.6 $\pm$ .4	.099 $\pm$ .003	71.2 $\pm$ 0.8
Methionine				
.67 (A)	10	9.7 $\pm$ .5	.107 $\pm$ .004	72.0 $\pm$ 1.3
.67 (R)	12	10.4 $\pm$ .4	.103 $\pm$ .003	72.7 $\pm$ 1.1
2.4 (R)	8	10.8 $\pm$ .1	.104 $\pm$ .003	71.1 $\pm$ 0.9
4.8 (R)	14	11.2 $\pm$ .6	.109 $\pm$ .004	70.3 $\pm$ 0.8
Thiouracil				
.12 (A)	10	8.6 $\pm$ .3	.091 $\pm$ .004	67.4 $\pm$ 1.0
.67 (A)	8	9.4 $\pm$ .3	.130 $\pm$ .010	69.5 $\pm$ 1.1
.67 (R)	12	9.3 $\pm$ .4	.106 $\pm$ .008	69.2 $\pm$ 1.2

Fleischman<sup>15</sup> have demonstrated that the creatine excretion of the thyrotoxic patient can be decreased by the administration of thiouracil. The data presented here demonstrate that the excretion of creatine is decreased in normal rats rendered hypothyroid by the administration of thiouracil. Methionine also demonstrated some tendency to decrease the excretion of creatine.

Thiouracil increases the size of the thyroid in rats fed the compound, (Table II). These data demonstrate that thiouracil not only increases the size of thyroid but also markedly increases the size of the liver. The increased size of livers in rats fed large doses of thioura-

cil has been previously reported by Leatham,<sup>5</sup> but his data was not correlated with a change in the nitrogen balance index.

Methionine, on the other hand, had no effect on the size of the liver but brought about a definite hypertrophy of the kidneys of rats fed high concentrations of this amino acid. Methionine in high level dosage also enlarged the thyroid. The enlargement of the thyroid upon the addition of methionine to the diet may be due to the high concentration of sulphur in the diet or to an antithyroid effect from the methionine. The changes in organ weights with both compounds are directly proportional to the dosage.

The nitrogen content of certain tissues of the body was altered by the addition of either

<sup>15</sup> Wilkins, L., and Fleischman, W., *J. Clin. Invest.*, 1946, **25**, 360.

TABLE I.

Excretion of Creatine in Rats Fed a Basal 12% Casein Diet to Which Was Added Varying Amounts of Thiouracil and Methionine. Experiments on a restricted food intake are followed by R and those in which the animals are fed *ad libitum* are indicated by A. The nitrogen balance index and the weight change over a 20-day period are included.

Dietary regime	No. of animals	Avg. wt. change 20-day period, g	N. balance index	Creatine, mg/day/kg
Control (R)	18	-17	.77	11.8 ± .3*
" (A)	14	50	.78	9.6 ± .3
Thiouracil				
.12 (R)	10	0	.85	8.7 ± .2
.67 (R)	8	-20	.58	5.6 ± .2
.67 (A)	12	+40	.58	3.0 ± .2
Methionine				
.67 (A)	10	56	.96	8.1 ± .1
.67 (R)	12	5	.96	9.0 ± .1
2.4 (R)	8	-40	.60	7.9 ± .1
4.8 (R)	14	-56	.60	9.2 ± .2

\*  $E = \frac{\sqrt{\sum d^2}}{\sqrt{N(N-1)}}$  Note: This equation for the standard error applies to all figures in the tables for which such data are given.

ration on a restricted caloric intake as shown by the open circles. The relationship between nitrogen balance and absorbed nitrogen is linear throughout the region of negative and of low positive nitrogen balance with an average nitrogen balance index of 0.77.<sup>11</sup> The recent work of Bricker and Mitchell<sup>12</sup> has confirmed this relationship in the rat.

The nitrogen balance index of the casein may be altered by adding certain compounds to the diet. The data in Table I demonstrate that the addition of 0.67% methionine (dry weight basis) to a casein diet increased the nitrogen balance index of casein in the rat from 0.77 to 0.96. The addition of larger amounts of methionine, however, decreased the nitrogen balance index markedly. The larger doses of methionine caused a definite restriction of food intake and necessitated the paired feeding of other groups to the methionine group. This restriction in food intake is paralleled by the weight change shown in Table I.

Thiouracil also had a marked effect on the nitrogen balance index of the casein. The addition of 0.6% thiouracil to the casein diet

gave a nitrogen balance index of 0.58. When .12% thiouracil was fed to rats on a restricted caloric intake, the nitrogen balance index was 0.85, and a value somewhat above that obtained with casein alone. Very high levels of thiouracil were not fed because Astwood<sup>13</sup> has reported that rats refuse diets containing high levels of the drug.

Despite the variation in nitrogen balance index produced by the addition of thiouracil or methionine to the casein diet, the straight line relationship between nitrogen balance and absorbed nitrogen is still valid. The data plotted in Fig. 2 demonstrate that the addition of methionine (0.6%) which increased the nitrogen balance index, and of thiouracil (0.6%) which decreased the nitrogen balance index did not alter the linearity of the relationship. The bending of the line at high nitrogen balance values is to be expected and has been reported by Allison and Anderson.<sup>7</sup>

The data in Table I demonstrate also that thiouracil decreases the excretion of creatine. The decrease in creatine excretion of the thyroidectomized animal has been reported by Allison and Leonard<sup>14</sup> and Wilkins and

<sup>11</sup> Brown, J. H., and Allison, J. B., Abstract 112th Meeting of Am. Chem. Soc., 1947, 51c.

<sup>12</sup> Bricker, H., and Mitchell, H. H., *J. Nutrition*, 1947, **34**, 491.

<sup>13</sup> Astwood, E. B., *J. Pharm. and Exp. Therap.*, 1943, **78**, 79.

<sup>14</sup> Allison, J. B., and Leonard, S. L., *Am. J. Physiol.*, 1941, **132**, 185.



FIG. 1.

Duodenum (D), gallbladder (G), and transplanted portion of the right uterine horn (arrow) are seen from the dog 20 weeks following operation (A left)  $\times 12$ . Cross section of the uterine horn enlarged (B right)  $\times 20$ .

grafting.

**Method.** Under intravenous nembutal anesthesia the abdomen was opened with a mid-line incision, the right uterine horn was clamped, and its proximal end ligated. The uterine artery was also ligated and severed at this level. The "broad ligament" was then freed by ligating its blood vessels along its posterior margin. The proximal 5 cm of the uterine horn was freed from the distal segment by applying a ligature and cutting proximal to it, preserving its blood supply from the ovarian artery. The common bile duct was ligated near its junction with the duodenum and severed distal to the ligature. The cut end of the common bile duct was then telescoped into the proximal end of the uterine horn while the opposite end of the segment was inserted into the duodenal wall through a longitudinal slit made about 2 cm anterior and medial to the choledochoduodenal junction. Both ends of the graft were secured in place by cotton sutures. The abdomen was closed with through and through interrupted cotton sutures.

Of 20 dogs surviving the operation, 12

died in from 3 to 21 days. The remaining 8 dogs were sacrificed between the 4th and the 27th weeks following operation: 2 in the 4th week and one each in the 6th, 14th, 16th, 20th, 24th, and 27th weeks. The abdominal organs of the latter animals were placed in 5% formalin and after fixation were studied grossly and microscopically.

**Results.** None of the 8 dogs that were killed 4 weeks or later had clay colored stools, or any evidence of jaundice in the sclerae or viscera. In each dog there were fairly dense adhesions about the site of operation involving the liver, stomach, duodenum, great omentum, and loops of small intestine. Numerous blood vessels of somewhat increased caliber from the ovarian artery appeared to supply the graft. The surface of the liver was smooth, glistening, and of the usual color. The gallbladder was distended with bile, and pressure on the viscus yielded bile in the duodenum at the site of implantation of the uterine horn.

The intrahepatic biliary ducts had spacious lumina. The hepatic ducts could not be clearly exposed. The common bile duct continued imperceptibly into the segment of

TABLE IV.

Influence of Various Concentrations of Methionine and Thiouracil on the Plasma Proteins of the Rat Fed a Basal Ration Containing 12% Casein.

Dietary regime	No. of animals	Albumin G, %	Globulin G, %	A/G	Hematocrit, %
Control (A)	18	3.10 $\pm$ .14	2.89 $\pm$ .14	1.10	48
" (R)	14	2.96 $\pm$ .22	2.86 $\pm$ .16	1.14	48
Thiouracil					
.12 (R)	10	3.20 $\pm$ .18	3.73 $\pm$ .18	0.86	46
.67 (A)	8	2.97 $\pm$ .16	3.74 $\pm$ .19	0.68	43
.67 (R)	12	3.00 $\pm$ .14	3.48 $\pm$ .17	0.67	43
Methionine					
.67 (R)	10	2.86 $\pm$ .19	3.69 $\pm$ .21	0.78	44
.67 (A)	12	2.81 $\pm$ .16	3.77 $\pm$ .19	0.74	45
2.4 (R)	8	2.89 $\pm$ .22	3.69 $\pm$ .18	0.78	47
4.8 (R)	14	3.02 $\pm$ .23	3.65 $\pm$ .19	0.82	46

thiouracil or methionine to the casein diet. The data presented in Table III demonstrate that methionine increased the nitrogen per gram of tissue of the liver and produced an over-all increase in liver-nitrogen. Thiouracil also increased the total liver nitrogen markedly, but the increase was due to the increased size of the organ rather than to the increased nitrogen per gram of tissue. Both methionine and thiouracil increased the globulin fraction of plasma with little change in albumin and decreased the A/G ratio (Table IV).

*Summary.* The addition of methionine and of thiouracil to the diet of rats altered the nitrogen balance index in proportion to the level of the compound in the diet. Although both methionine in high concentration and thiouracil caused a decrease in body weight and a reduction in nitrogen balance index, the

compounds increased the total liver nitrogen; however, methionine increased the nitrogen per gram of tissue without affecting the size of the organ while thiouracil increased the size of the organ without affecting the nitrogen per gram of tissue. Both methionine and thiouracil increased the size of the thyroid, while methionine alone increased the size of the kidney. Both compounds increased the total plasma proteins by increasing the plasma globulin. Both methionine and thiouracil decreased creatine excretion; however, the effect of thiouracil is marked in comparison to methionine. These data indicate that the result of feeding methionine or thiouracil is very similar in end result but that the mechanism may not be identical because the results are reached through different pathways.

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## 17274. Replacement of a Portion of the Common Bile Duct with a Segment of Uterine Horn.

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Attempts at repair are comparatively unsuccessful when the common bile duct is accidentally so injured that an end-to-end anastomosis is not feasible. The present studies were undertaken to ascertain whether a graf-

ted tubular structure with independent blood supply would maintain its viability and carry the bile from the common bile duct to the duodenum. Dogs were selected for the experiments and the uterine horn was used for

used for the grafting. Of 20 dogs surviving the operation, 12 died in from 3 to 21 days, and the rest were sacrificed between the 4th and the 27th weeks following the operation. Gross and microscopic studies of the biliary system of the latter 8 dogs disclosed that, in

all, the transplanted segment of the uterine horn connected the common bile duct and the duodenum without causing permanent severe damage to the biliary system.

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### 17275. Observations on Experimental Aortic Anastomosis.

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(Introduced by J. C. Hinsey.)

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The following report is based on observations made upon 3 dogs that were subjected to end-to-end anastomosis of the thoracic aorta at the age of 6 weeks and then studied approximately one year after operation.

*Method of Study.* Six young dogs, male and female litter mates, were subjected to division of the aorta and end-to-end anastomosis at the site of division at ages between 6 and 8 weeks. A seventh male dog was op-

erated on in a similar manner at the age of 3 weeks. The operations were performed under intravenous nembutal anesthesia and endotracheal insufflation. The anastomosis was accomplished with a single continuous everting mattress suture of No. 00000 arterial silk attached to an atraumatic needle.

Four of these dogs died 22 to 42 days after operation from intestinal infestation. Autopsy in each case revealed the

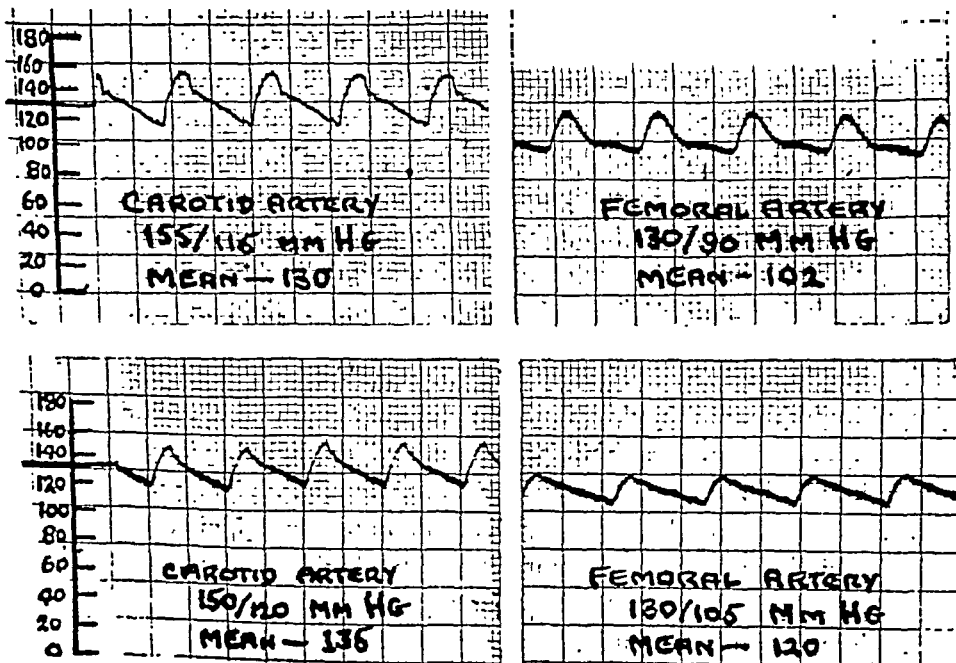


FIG. 1.



FIG. 2.

Duodenum (D) and transplanted portion of the right uterine horn are seen from the dog 6 weeks following operation (A left)  $\times 12$ . Cross section of the uterine horn enlarged (B right)  $\times 20$ .

uterine horn that connected it with the lumen of the duodenum.

After completion of the gross studies, microscopic sections were prepared of the liver, gallbladder, transplanted uterine segment, duodenum, and left uterine horn.

In the liver there were chronic cholangitis and pericholangitis, evidenced by an infiltration with lymphocytes, plasma cells, and large mononuclear cells in the wall of the bile ducts and in the surrounding portal connective tissue. The liver lobules were somewhat diminished in size and in their central portions there was some slight increase of the connective tissue and a decrease in size of the liver cells. Many of the bile capillaries contained inspissated secretion. In the gallbladders of some of the dogs there was slight to moderate chronic cholecystitis with focal and diffuse infiltrations of the tunica propria with chronic inflammatory cells. In all of the animals the implanted segment of the uterine horn carried the bile from the common bile duct to the duodenum. In some instances, the columnar epithelium covering the surface was almost intact. In others, the

epithelium was intact only in the fundi of the glands and a cellular debris covered granulation tissue that partly replaced the bile stained mucosa. Invariably the muscular coats appeared intact (Fig. 1 and 2).

*Comment.* There is no submucosa in either the common bile duct or the uterine horn of the dog. Of the 2 the uterine horn is the sturdier structure. In the human, also, the common bile duct and the Fallopian tube have no submucosa. Of these the Fallopian tube is usually the more delicate structure. These experiments were conducted, not to suggest that the Fallopian tube be used as a pedicle graft, but rather to ascertain whether a grafted tubular structure with independent blood supply would serve for the passage of bile from the common bile duct to the duodenum. The evidence herein presented proved that this can be accomplished.

*Summary.* Experiments were conducted to ascertain whether a grafted tubular structure with independent blood supply would serve for the passage of bile from the common bile duct to the duodenum. Dogs were selected for the experiments and the uterine horn was

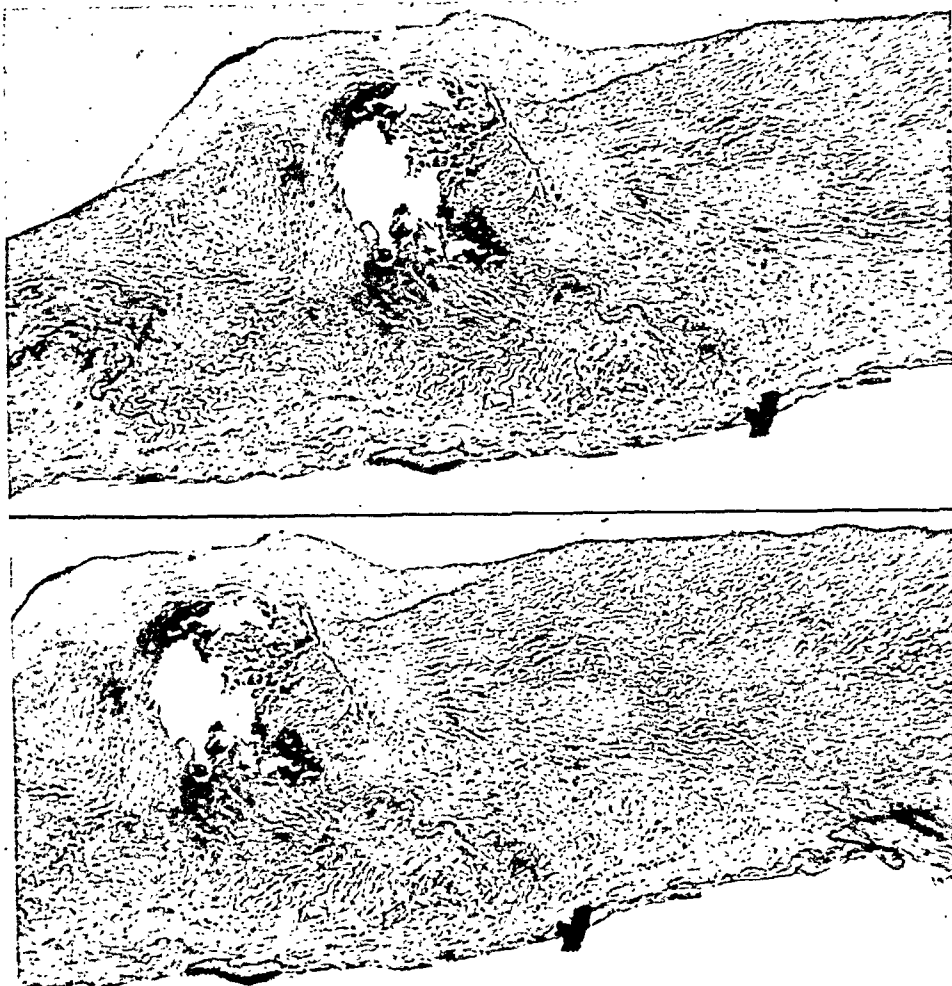


FIG. 5.

Photomicrograph of segment of aorta removed from dog 795. Longitudinal section through the site of anastomosis. In the upper photograph the site of suture is shown in the center of the photograph, the intimal surface being on the superior surface of the section. In the lower photograph the section has been moved to the left in order to show the adjacent aortic wall.

tion at the suture line but atheromatous changes were absent. (Fig. 5) Foreign body reaction was apparent immediately adjacent to the silk suture and there was scarring at the junction. The aortic wall to either side, however, was well preserved. Elastic tissue stains showed good preservation of the media.

*Discussion.* Interest in surgical intervention of coarctation of the aorta has been active since the report by Crafoord and Nylin<sup>1</sup>

of successful excision of the involved segment of aorta and anastomosis. Because the average age at death of 74% of the people afflicted with this congenital lesion has been shown to be 30 years,<sup>2</sup> it is felt that operation should be performed before maturity. In addition, atheromatous changes in the aorta occur early in association with this malformation and accordingly it is technically advantageous to undertake operation at a relatively early age before such changes take place.

<sup>1</sup> Crafoord, C., and Nylin, G., *J. Thoracic Surg.*, 1945, 14, 347.

<sup>2</sup> Gross, R. E., *J.A.M.A.*, 1949, 139, 285.





FIG. 2.

Dog 795. Angiocardiogram obtained with 16 cc of neo-iopax. (Retouched).



Fig. 3 (left). Dog 797. Angiocardiogram, using 10 cc of 70% diodrast, shows narrowing of thoracic aorta at site of anastomosis. There appears to be some dilatation of the arch of the aorta. (Retouched).

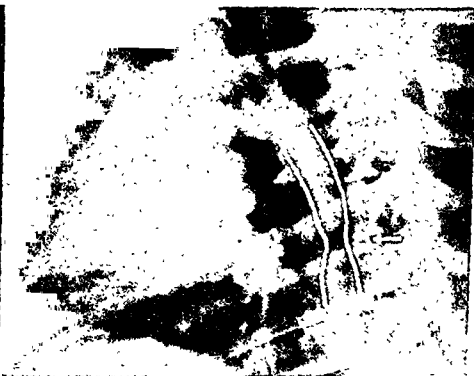


Fig. 4 (right). Dog 798. Angiocardiogram showing slight constriction of thoracic aorta. 14 cc of neo-iopax was used for contrast substance. (Retouched).

suture line to be intact and without evidence of mural thrombosis. The 3 remaining dogs, one male and 2 females, developed normally and at the time of these investigations weighed 28, 34, 34½ lb respectively.

Approximately one year after operation, angiocardigraphic studies were made. Under intravenous sodium pentobarbital anesthesia, the left external jugular vein was cannulated. Fourteen to 16 cc of neo-iopax was given rapidly through the cannula in 2 cases, and 10 cc of diodrast in the third. Roentgenograms were obtained at 0.5 sec. intervals throughout the cardiac cycle until the thoracic aorta had been visualized.

Femoral pulses were palpable in all 3 animals and blood pressure recordings were obtained with a Sanborn electromanometer in the carotid and femoral arteries of 2 of the dogs. (Fig. 1 and Table I)

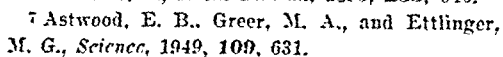
Eleven days after angiocardigraphy, the male dog was subjected to thoracotomy and the site of anastomosis removed. The specimen was submitted to the Department of Surgical Pathology where sections were made longitudinally through the site of anastomosis for microscopic study.

*Analysis.* Angiocardiographic studies demonstrated in each animal a definite narrowing at the site of anastomosis. (Fig. 2, 3, 4) In one there appeared to be a slight dilation of the aortic arch (Fig. 3); however, there was

no evidence of aneurysmal dilation either above or below the narrow area. There was no suggestion of compensatory collateral circulation in any case.

In the dog subjected to thoracotomy, the area of constriction was readily visualized (Fig. 4). The diameter of the aorta was approximately the same above and below the site of stenosis. It also appeared that the lumen of the anastomotic site was larger than the lumen of the aorta had been when the dogs were 6 weeks of age. The silk suture was still present. (Fig. 5)

Microscopic examination of the resected segment of aorta revealed an intimal prolifera-



The first case reported by Gross was a 5-year-old boy<sup>3</sup> and, in discussing his experience with 60 cases recently, whose ages ranged from 5 to 30 years, he stated that operation should be avoided before 6 to 8 years of age.<sup>2</sup> Gross indicated that the risks in babies and very young children are high<sup>3</sup> because collateral channels are not well developed and because the aorta is too small for the anastomosis to be accomplished with facility. He stated further than "an aortic lumen may be established at the anastomosis which is satisfactory for a child of a few years of age, but it will probably be insufficient in size when the person grows to maturity."

The experiments reported in this paper appear to have some bearing on the latter two points. There may be some doubt as to the necessity of delaying operation until a child is 6 to 8 years old to assure that the aorta will be large enough to suture without excessive risk. The dogs in this experiment were under 5 lb in weight and less than 8 weeks old at the time of operation, and yet the technical aspects of the anastomoses were

accomplished without mishap. The 4 fatalities in the postoperative period were unrelated to the operative procedures.

On the other hand, it would appear that the diameter of the lumen at the site of anastomosis in these dogs did not keep pace in growth with the rest of the aorta. This would support the view that surgical intervention for coarctation should be deferred until the lumen of the aorta is large enough in diameter to insure adequate size as the individual grows to maturity. To be sure, a single continuous suture was used for the anastomosis which might have some bearing on the formation of the constriction.

*Summary.* Seven young dogs between the ages of three and eight weeks were subjected to end-to-end anastomosis of the thoracic aorta. Three of these dogs survived and have been studied approximately one year after operation to determine the status of the site of anastomosis.

Narrowing of the aortic lumen at the suture line was demonstrated in each animal by angiocardigraphic methods and also in one at thoracotomy.

<sup>3</sup> Gross, R. E., *Surgery*, 1945, **13**, 673.

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## 17276. Isolation of an Anti-thyroid Compound from Rape Seed (*Brassica Napus*).

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In a study of the toxic reactions produced in rats by phenylthiourea and  $\alpha$ -naphthylthiourea (ANTU), it was found that rats which had been pretreated with anti-thyroid compounds or fed on certain goitrogenic diets developed a marked resistance to these toxic compounds.<sup>1</sup> This effect has been used as an assay method in the extraction of a goitrogenic compound from rape seed.

*Materials and methods.* Adult Sprague-Dawley rats of either sex were used in assay-

ing the rape seed fractions. Crude fractions were tested by mixing them with the normal diet of fox chow and feeding for 4 days, following which the rats were injected intraperitoneally with 30 mg/kg of phenylthiourea. More refined fractions were dissolved in water and given in a single subcutaneous injection, followed 24 hours later by 20 mg/kg of phenylthiourea intraperitoneally. Survival of the rats for more than 48 hours after the toxic dose was used as the criterion of activity in the extracts.

The normal LD 50 of phenylthiourea for

<sup>1</sup> Carroll, K. K., and Noble, R. L., *Fed. Proc.*, 1949, **8**, 22.

TABLE I.  
Effect of Administration of Various Amino Acids, Saline and Sodium Bicarbonate on Level of Glutamine in Blood Plasma.

Rabbit No.	Wt., kg	Substance administered	Amino acid nitrogen in plasma, mg per 100 ml			Glutamine in plasma, mg per 100 ml		
			Before	30 min.	60 min.	Before	30 min.	60 min.
1	3.1	dl-alanine	7.0	14.4	10.2	6.0	8.9	6.8
2	3.4		5.9	14.8	12.7	7.2	10.4	10.6
3	3.5		6.5	16.8	13.4	7.1	9.4	8.4
4	4.0		8.4	19.0	13.2	8.8	12.7	10.5
5	4.4		6.9	14.5	12.8	7.3	11.1	9.0
6	4.0		6.8	14.7	11.5	7.5	11.2	10.3
Average	3.73		6.9	15.7	12.3	7.3	10.6	9.3
				(8.8)*	(5.4)	Fisher's "t" P	13.6 <0.01	6.43 <0.01
7	3.9	glycine	5.2	17.4	14.4	6.1	8.3	8.3
8	3.9		7.4	18.2	15.3	7.5	9.3	9.5
9	4.1		7.7	18.7	15.5	8.1	9.6	9.3
10	3.8		6.9	16.8	15.2	11.4	12.5	10.9
11	3.6		6.6	14.1	10.5	7.4	7.7	8.2
12	4.1		6.6	14.6	11.5	8.0	10.1	8.7
Average	3.9		6.7	16.6	13.7	8.1	9.6	9.2
				(9.9)	(7.0)	Fisher's "t" P	4.67 <0.01	4.34 <0.01
13	3.4	$\beta$ -alanine	7.8	19.5	17.1	7.6	8.1	8.3
14	4.0		6.3	21.2	16.7	7.4	8.1	9.3
15	4.2		6.1	19.5	14.9	5.8	8.5	8.0
16	4.3		7.1	21.1	17.6	9.5	10.6	10.1
17	4.2		6.9	21.7	16.3	8.7	8.5	8.7
18	3.6		5.9	21.2	18.9	8.4	9.2	8.8
Average	3.95		6.7	20.7	16.9	7.9	8.5	8.9
				(14.0)	(10.2)	Fisher's "t" P	2.35 >0.05	3.01 <0.05
19	4.4	Sodium bicarbonate	6.6	6.6	6.4	8.0	7.8	6.5
20	4.2		7.4	6.8	6.6	8.4	7.6	8.1
21	3.5		7.2	6.8	6.6	7.0	7.7	8.5
22	4.0		6.9	6.2	—	9.8	8.6	8.7
23	4.4		7.2	7.2	7.1	7.6	7.8	8.0
24	3.8		6.4	7.0	7.3	8.3	7.2	8.8
Average	4.05		6.7	6.8	6.8	8.2	7.8	8.1
				(0.1)	(0.1)	Fisher's "t" P	1.71 >0.1	0.160 >0.8
25	3.9	Saline	6.4	6.3	6.0	6.6	7.0	6.1
26	4.1		6.7	6.1	6.3	7.7	7.1	7.7
27	4.5		6.4	6.3	6.3	6.5	6.3	5.6
28	3.9		6.9	6.8	6.9	6.9	7.2	7.4
29	2.4		5.9	5.5	6.1	5.0	5.1	5.5
30	3.5		5.9	6.4	5.7	5.9	6.0	6.0
Average	3.72		6.4	6.2	6.2	6.4	6.4	6.4
				(0)	(0)	Fisher's "t" P	.206 >0.8	.096 >0.9

\* The figures in parentheses represent the increments, above the initial level, of the average level of amino acid nitrogen per 100 ml of plasma.

small number of experimental animals showed that the increments obtained were highly significant for both periods with  $P < 0.01$ .

*Glycine.* The administration of glycine produced an average rise in the level of glutamine of 1.5 mg for the 30 minute period and

Their experiments indicated that the anti-thyroid activity of this compound in humans is comparable to that of 6-n-propylthiouracil.

*Summary.* A new method for the detection of compounds which have anti-thyroid

activity is described. This method has been used to isolate a crystalline anti-thyroid compound from rape seed having the empirical formula  $C_8H_7ONS$ .

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### 17277. Effect of Amino Acids and Sodium Bicarbonate on the Level of Glutamine in Blood. V.

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In a previous publication<sup>1</sup> we reported on the depressing effect of glucose and insulin administration on the level of glutamine and amino acids in the blood plasma and some of the possible mechanisms involved in producing this effect were discussed. In a subsequent publication<sup>2</sup> it was shown that this was not due to a mere diffusion of amino acids and glutamine into the tissues as had been suggested by Hamilton.<sup>3</sup>

In this paper we wish to report the effect of the intravenous administration of some amino acids, sodium bicarbonate and physiological saline solution on the level of glutamine in the blood plasma of rabbits.

*Procedure and methods.* Male rabbits, 18 hours post absorptive, were used for these experiments. Six animals were used for each substance which was tested. The amino acids and sodium bicarbonate were made up as a 10 percent solution in distilled water and 10 ml or the equivalent of 1 g of substance per 3 kg of body weight was administered intravenously. The saline solution was administered in quantities of 10 ml. Since the rabbits were very similar in weight (Table I) the volumes varied by only one or two ml. The rabbits were bled from the marginal vein of the ear just before injection and 30 and 60

minutes after injection. About 12 ml of blood were drawn at each bleeding into tubes containing sodium oxalate to prevent coagulation. The blood was immediately centrifuged for about 10 minutes and the plasma was used for the determination of total amino acids and glutamine. The amino acids were determined colorimetrically by Russell's modification of Frame's method<sup>4</sup> and the glutamine by our method as described in a previous publication.<sup>5</sup> The determinations were carried out in duplicate or triplicate and statistical analysis of the data showed that the variations between duplicate determinations were negligible.

*Observations.* It will be seen from Table I that the initial level of glutamine in the post-absorptive state bore no relation to the level of the total amino acids during this period.

*dl-Alanine.* Following the intravenous administration of dl-alanine there was a marked rise in the level of glutamine in the first 30 minutes. This amounted to an average increment of 3.3 mg per 100 ml of blood plasma. Although the level tended to decrease in the following 30 minutes it still was above the initial level by an average of 2.0 mg. Statistical analysis by Fisher's "t"<sup>6</sup> method for a

<sup>1</sup> Harris, M. M., Roth, R. T., and Harris, R. S., *J. Clin. Invest.*, 1943, **22**, 577.

<sup>2</sup> Harris, M. M., and Harris, R. S., *Proc. Soc. Exp. Biol. and Med.*, 1947, **64**, 471.

<sup>3</sup> Hamilton, P. B., *J. Biol. Chem.*, 1945, **158**, 397.

<sup>4</sup> Russell, J. A., *J. Biol. Chem.*, 1944, **155**, 467.

<sup>5</sup> Harris, M. M., Roth, R. T., and Harris, R. S., *J. Clin. Invest.*, 1943, **22**, 569.

<sup>6</sup> Mainland, D., *Treatment of Clinical and Laboratory Data*, p. 147, Oliver and Boyd, London, 1938.

The results of both methods of analysis (unadjusted averages and "adjusted means") were essentially similar. (Table II)

It will be noted that the most marked effect was obtained from the administration of dl-alanine and lesser effects were produced by glycine and  $\beta$ -alanine in this order. Since the average values for the initial level of glutamine of the groups of animals used for the administration of sodium bicarbonate and saline, which may be considered as the controls, consisted of both a high and low average value, this also acted as a control for the effect of the variation in the initial level of glutamine.

**Discussion.** It will be noted that the increment in the level of the amino acid nitrogen after 30 and 60 minutes remained much higher following the administration of  $\beta$ -alanine than that of dl-alanine. This probably indicates that the latter entered the metabolic processes of the tissues more rapidly and is in keeping with the more marked effect on the level of glutamine produced by it.

Christensen and his coworkers<sup>9</sup> have reported that the oral administration to guinea pigs of various  $\alpha$ -amino acids, except glutamic acid, will produce a rise in glycine in the plasma with a decrease in the ratio of the concentration of glycine in the liver and muscles to that in the plasma. This was interpreted by them as due to competitive inhibition between some of the amino acids for the means by which the cells concentrate amino acids. However, from a recalculation of their data it is apparent that although the distribution ratio for glycine fell the actual concentration of glycine in the liver cells rose above the fasting level in some cases. It would seem that the rise in the level of glycine in the plasma could be interpreted as resulting from a marked production or accumulation of glycine accompanied by an increased burden placed upon the concentrating power of the liver cells. The authors give no data regarding glutamine except that following the administration of glutamate which resulted in an increase in glutamine production.

If the claims of Christensen and his co-workers are correct, it is possible that the rise in the level of glutamine in the plasma may be due to a displacement of glutamine from the tissues by dl-alanine or glycine. We are inclined to believe, however, that this is probably due to an increased production of glutamine. 1-Alanine is known to play an important role in the transamination of glutamic acid to 1-alanine (glutamic acid + pyruvic acid  $\leftrightarrow$  alanine + ketoglutaric acid). An increase in the supply of alanine would tend to reverse this reaction and thus hinder transamination and favor amidation. Braunstein<sup>10</sup> has indicated that there is an interrelation between transamination and amidation and where transamination is decreased amidation or glutamine formation is increased and vice versa. Since glycine, as far as is known, does not enter into transamination mechanisms the process whereby it produces a rise in the level of glutamine requires further elucidation. It is possible that it may result from the amidation of free ammonia liberated by the amino oxidase of glycine. Various investigators<sup>10,11</sup> have indicated that amidation is probably an important means whereby ammonia is rapidly fixed by the formation of glutamine in the animal organism.

In connection with our observations it may be of interest to call attention to the recent findings of Awapara and his coworkers<sup>12</sup> that the dicarboxylic amino acids are decreased and alanine is increased in the liver of adrenalectomized rats given 17 hydroxydehydrocorticosterone (Compound E of Kendall).

**Summary.** Studies were carried out regarding the effect of the intravenous administration of dl-alanine, glycine,  $\beta$ -alanine, sodium bicarbonate and saline on the level of glutamine in the blood of rabbits. dl-Alanine was found to produce a marked rise in the level of glutamine. Glycine produced a smaller and  $\beta$ -alanine the smallest rise. Sodium bicar-

<sup>10</sup> Braunstein, A. E., *Advances in Protein Chemistry*, Vol. 3. p. 1, Academic Press, Inc., New York, 1947.

<sup>11</sup> Krebs, H. A., *Biochem. J.*, 1935, **29**, 1951.

<sup>12</sup> Awapara, J., Marvin, H. N., and Wells, B. B., *Endoc.*, 1949, **44**, 378.

<sup>9</sup> Christensen, H. N., Streicher, J. A., and Elbinger, R. L., *J. Biol. Chem.*, 1948, **172**, 515.

1.1 mg for the 60 minute period. The "t" values for both periods were significant with  $P < .01$ . Only one of the 6 animals, (No. 10) showed a slight drop below the initial level in the 60 minute period.

**$\beta$ -Alanine.** Following the administration of  $\beta$ -alanine there was an average rise in the level of glutamine of 0.6 mg in the 30 minute period and 1.0 mg after 60 minutes. The "t" value was not significant for the 30 minute period but was significant for the 60 minute period. The value of P was greater than 0.05 for the former and less than 0.05 for the latter period.

**Sodium Bicarbonate and Saline.** There was a slight drop in the average level of glutamine following the administration of sodium bicarbonate and no change following saline. The "t" values, however, were not significant for any of the periods.

**Total Amino Acids Nitrogen.** The average values of the total amino acid nitrogen in the different groups were very similar in the initial period before injection. Following the injection of saline and sodium bicarbonate the average values remained practically unchanged in the 30 and 60 minute periods. The administration of the various amino acids resulted in a rise in the level of the total amino acid nitrogen in the plasma which remained elevated with a tendency to fall in the second 30 minute period. The smallest rise occurred with dl-alanine and the highest rise occurred with  $\beta$ -alanine.

**Statistical Analysis.** The data were analyzed by Fisher's "t" method and the P values for significance were determined from Fisher's table. Any values of P equal to or less than 0.05 were considered significant.

Owing to the fact that the initial average level of glutamine in the plasma was not the same for the various groups, the data were analyzed by the method of variance and covariance by which statistically "adjusted means"<sup>7,8</sup> are obtained.

TABLE II.

Changes in the Level of Glutamine in Blood Plasma Resulting from the Administration of Various Substances Compared with Statistically Adjusted Results by the Method of Variance and Covariance.

Substance administered	Obtained average values of glutamine in plasma, mg per 100 ml				Statistically adjusted mean values of glutamine in plasma, mg per 100 ml			
	Before	30 min.	60 min.	Increment	Before*	30 min.	60 min.	Increment
dl-alanine	7.3	10.6	9.3	3.3	7.58	10.81	9.46	3.23
Glycine	8.1	9.6	9.2	1.5	7.58	9.20	8.90	1.62
$\beta$ -alanine	7.9	8.5	8.9	1.1	7.58	8.27	8.71	1.13
Sod. bicarb.	8.2	7.8	8.1	-0.4	7.58	7.33	7.74	0.16
Saline	6.4	6.4	6.4	0.0	7.58	7.30	7.09	-0.23
Average	7.58	8.58	8.38	1.0	7.58	8.58	8.38	1.0
F	1.8	12.1	7.5			29.0	8.6	
P	>0.05	<0.01	<0.01			<0.01	<0.01	

\* In order to equate for initial level, it is assumed that all of the individual animals showed a "before" average of 7.58 equal to the grand average of all the animals in the "before" determination.

<sup>7</sup> Rider, P. R., Introduction to Modern Statistical Methods, Analysis of Covariance, p. 150, John Wiley and Sons, Inc., New York, 1939.

<sup>8</sup> Snedecor, G. W., Statistical Methods, p. 116, 215, 318, Iowa State College Press, Ames, Iowa, 1946.

TABLE I.  
Effect of Administration of Sodium Benzoate on the Level of Glutamine in the Blood.

Rabbit No.	Wt., kg	Substance administered*	Time, min.	Glutamine in plasma, mg per 100 ml	Amino acid nitrogen in plasma, mg per 100 ml	Blood sugar, mg per 100 ml
1	2.5	0.25 g benzoic acid in 8 ml water	0	8.0	9.4	132
			30	6.3	8.7	155
			60	6.8	9.1	220
2	2.6	0.25 g benzoic acid in 8 ml water	0	7.9	10.0	118
			30	5.9	9.7	124
			60	6.0	10.1	152
3	2.9	0.25 g benzoic acid in 5 ml water	0	8.6	9.9	126
			30	6.5	9.8	218
			60	6.6	9.2	260
4	2.9	0.5 g benzoic acid in 10 ml water	0	7.5	10.5	118
			30	6.5	9.8	148
			60	7.5	10.0	172
5	2.9	0.75 g benzoic acid in 10 ml water	0	8.0	8.0	113
			30	7.3	7.3	122
			60	6.9	6.9	119
6	2.9	0.75 g benzoic acid in 10 ml water	0	8.3	7.8	122
			30	7.3	7.3	134
			60	6.9	7.3	163
7	4.2	2.0 g in gelatin capsules + 15 ml water (orally)	0	8.1	7.2	—
			75	5.7	7.1	95
			175	6.1	7.4	96
Human subject 59.1		5.9 g sod. benzoate in capsules + 200 ml water	0	6.6	9.1	89
			45	7.0	8.5	77
			85	6.0	8.4	84
			135	4.8	8.7	92
8		No treatment	0	8.3	9.0	
			30	8.4	9.0	
			60	8.1	9.4	
		Saline controls, avg. for 6 animals see previous paper	0	6.4	6.3	101
			30	6.5	6.2	105
			60	6.5	6.2	109

\* Sodium benzoate was administered intravenously except where otherwise indicated.

Statistical analysis of the effect of the intravenous administration of sodium benzoate gave a Fisher "t" value of 6.64 and 6.30, with  $P < 0.01$  for the changes in the level of glutamine for the 30 and 60 min. periods respectively, which is highly significant. The corresponding changes in the level of amino acid nitrogen gave a Fisher "t" value of 2.03 with  $P > 0.05$  and 1.81 with  $P > 0.1$  which were not statistically significant.

in some of the experiments that the rise was due in part to an increase in a reducing substance which did not undergo glycolysis and was undoubtedly due to glycuronic acid.

Sodium benzoate was administered orally in capsules to one rabbit and one human female subject in doses of 2.0 g and 5.9 g respectively. The level of glutamine dropped markedly in both cases. However, the level of amino acid nitrogen remained practically unchanged in

the rabbit. In the human subject the glutamine level dropped 27% and the total amino acid nitrogen only about 4% at the end of 135 min. after the administration of sodium benzoate. The "blood glucose" showed very little change in both cases and in fact was slightly lower in the human subject after 45 minutes.

*Discussion.* It is claimed that benzoic acid is detoxified by forming a glycuronide in the



bonate and saline produced no rise in the average level of glutamine in the group of animals.

The authors are indebted to Dr. Joseph Zubin for his advice and aid in the statistical analysis of the data.

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## 17278. Effect of Administration of Sodium Benzoate on the Level of Glutamine in Blood. VI.

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In previous publications we reported on the effect of the administration of insulin, glucose,<sup>1</sup> amino acids and sodium bicarbonate<sup>2</sup> on the level of glutamine in the blood. It was suggested that the effect of insulin in lowering the level of glutamine in the blood might be due, in part, to the depression of oxidative deamination of amino acids by insulin. Since it has been reported that benzoic acid depresses the oxidation of d-amino acids<sup>3</sup> and other intermediary metabolites<sup>4,5</sup> the effect of its administration on the level of glutamine was investigated.

*Procedure and methods.* Male rabbits in the post absorptive state were used for these studies. The effect of the administration of sodium benzoate was studied also in a normal human female subject. The benzoate was administered either intravenously or orally as indicated in the table. Blood was collected in tubes containing sodium oxalate and the plasma separated by centrifugation. Glutamine was determined by the method described in a previous publication.<sup>6</sup> The total amino acids were determined by the method of Si-

monelli.<sup>7</sup> The blood sugar (total reducing substance) was determined by the Folin-Wu method<sup>8</sup> which includes other non-glucose reducing substances. In some cases the blood was allowed to glycolize at 37°C overnight in order to determine the change in non-glucose reducing substances.

*Observations.* Doses of 0.25 g, 0.5 g, and 0.75 g of benzoic acid neutralized with sodium hydroxide and injected intravenously produced a fall in the level of glutamine in the plasma both in the 30 min. and 60 min. period after injection. The extent of the fall varied in different animals and was not related to the size of the dose of benzoic acid. Statistical analysis of the fall showed that the drop was statistically significant for both periods (see table).

The level of the total amino acid nitrogen in the blood plasma tended to fall in all animals; however, this was negligible in some animals. The extent of the change in the level of glutamine was not parallel with the changes in total amino acid nitrogen. The blood sugar (total reducing substances) also rose in all the animals which received benzoic acid intravenously. The extent of the rise also was variable and two of the animals which showed the most marked rise (Rabbits nos. 1 and 3) in the "blood sugar" received the smallest dose of benzoic acid. By incubating the blood at 37°C overnight it was found

<sup>1</sup> Harris, M. M., Roth, R. T., and Harris, R. S., *J. Clin. Invest.*, 1943, **22**, 577.

<sup>2</sup> Harris, M. M., and Harris, R. S., *Proc. Soc. Exp. Biol. and Med.*, preceding paper.

<sup>3</sup> Klein, J. R., and Kamin, H., *J. Biol. Chem.*, 1941, **138**, 507.

<sup>4</sup> Griffith, W. H., *Proc. Soc. Exp. Biol. and Med.*, 1937, **37**, 279.

<sup>5</sup> Jowett, M., and Quastel, J. H., *Biochem. J.*, 1935, **29**, 2143 and 2159; Quastel, J. H., and Wheatley, A. H. M., *ibid.*, 1935, **29**, 2773.

<sup>6</sup> Harris, M. M., Roth, R. T., and Harris, R. S., *J. Clin. Invest.*, 1943, **22**, 569.

<sup>7</sup> Simonelli, U., *Clinical Colorimetry with the Pulfrich Photometer*, by W. Krebs, p. 24, Carl Zeiss, Jena.

<sup>8</sup> Folin, O., and Wu, H., *J. Biol. Chem.*, 1920, **41**, 367.

# 17279. Aureomycin in Experimental Polyarthrititis with Preliminary Trials in Clinical Arthritis.\*†

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A polyarthrititis of rats can readily be reproduced by intraperitoneal or intravenous injection of broth cultures of the L<sub>4</sub> strain of pleuropneumonia-like organism. This experimental polyarthrititis has been used as a means of making chemotherapeutic trials.<sup>1-3</sup> In man pleuropneumonia-like organisms have repeatedly been isolated from the genitourinary tract and "may be related etiologically to an acute infectious type of arthritis and to Reiter's syndrome."<sup>4</sup> Dienes has isolated L type cultures from many Gram negative bacilli and several large Gram positive bacilli,<sup>5</sup> although thus far no one has reported isolation of such forms from streptococci. Unfortunately these microorganisms are species specific as far as their pathogenicity is concerned so that human strains do not produce infections in animals. While the polyarthrititis of rats is not the same disease as rheumatoid arthritis in man, the course of both is favorably altered by the use of gold salts. In the rat the arthritis may be prevented by the intramuscular injection of gold at the time of infection, or after the arthritis is developed, treatment with gold will promote healing more

rapidly than in untreated controls.<sup>1</sup> Our effort in the chemotherapy of this rat arthritis has been directed toward finding agents which might be as effective as gold, but less toxic, for possible trials in rheumatoid arthritis of man. The new antibiotic, aureomycin, has proved to be successful in this experimental polyarthrititis.

**Methods.** Aureomycin was given to the infected rats by stomach tube, by subcutaneous injection, or by mixing with the diet. The effect of aureomycin both as a preventive and a curative agent was evaluated. Scoring was designed to include the per cent incidence of arthritis in each group of white rats tested, and the per cent survival. The extent of the joint involvement was evaluated according to a modification of the arthrogram of Sabin and Warren<sup>6</sup> which assigns a numerical value of 4 to each front leg and 5 to each hind leg, giving a total of 18 points per animal assuming maximal joint involvement of all joints. The average arthrogram scores were determined by adding the total scores for each group and dividing by the number of animals in the group irrespective of per cent incidence of arthritis.

The microbe was cultured according to a method described by Tripi and Kuzell<sup>1</sup> in a broth culture which is a buffered yeast-extract tryptose base enriched with 20% filtered horse serum. Table I shows the protective effect of aureomycin given coincident with the intraperitoneal injection of 2 cc of a broth culture of the microorganism or shortly thereafter.

**Results.** When 0.1% or 0.3% aureomycin was mixed with the ground Purina dog chow complete protection of all animals occurred (Table I). When aureomycin was adminis-

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† The aureomycin was supplied by Lederle Laboratories Division of the American Cyanamid Company, Pearl River, N. Y.

<sup>1</sup> Tripi, H. B., and Kuzell, W. C., *Stanford Med. Bull.*, 1947, 5, 98.

<sup>2</sup> Findlay, G. M., Mackenzie, R. D., and MacCallum, F. O., *Brit. J. Exp. Path.*, 1940, 21, 13.

<sup>3</sup> Sabin, A. B., and Warren, J., *Science*, 1940, 92, 535.

<sup>4</sup> Dienes, L., Ropes, M. W., Smith, W. E., Madoff, S., and Bauer, W., *New England J. Med.*, 1948, 238, 509 and 563.

<sup>5</sup> Dienes, L., *Proc. Soc. Exp. Biol. and Med.*, 1949, 71, 30.

<sup>6</sup> Tripi, H. B., Gardner, G. M., and Kuzell, W. C., *Proc. Soc. Exp. Biol. and Med.*, 1949, 70, 45.

rabbit and hippuric acid in man. If this is so, it would mean that the level of glutamine is depressed irrespective of the mode of detoxification used by the animal organism. It is interesting to note the variable effect of the administration of benzoic acid on the level of the total amino acids and the total reducing substances in the blood. Although glycine was withdrawn from the metabolic mixture as hippuric acid in the experiment on the human subject (several grams of crystalline hippuric acid were isolated from her urine), still the level of the total amino acids in the blood was lowered to no greater extent than in some of the rabbits. These variable effects in the different animals suggest that variable metabolic changes, besides that involved in the formation of benzoyl glycuronide, probably occur in different rabbits. The fall in the level of glutamine may be due, in part, to the inhibition of the oxidative deamination of amino acids beside the inhibition of other oxidative processes.<sup>4,5</sup> This would reduce the supply of nitrogen for amidation. However, where there was a fall in the level of the total amino acids in the blood it is possible that certain amino acids were withdrawn from the metabolic mixture thus reducing the supply of nitrogen for amidation. The inhibition of deamination alone should tend to raise the level of the amino acids in the blood. Since in some cases the level of glutamine was depressed without any significant change in the level of the total amino acids (Rabbits nos. 2, 3 and 7) it is possible that the process of amidation was directly depressed.

Christensen and his co-workers<sup>9</sup> claim that special mechanisms are present in cells for

concentrating amino acids and that in the presence of high concentrations of amino acids there is competition between the various amino acids for these mechanisms. If the depression in the level of glutamine in the plasma were due to a shift of glutamine into tissue cells due to the removal of glycine by conjugation with benzoic acid then one should obtain a significant drop in the level of the total amino acid nitrogen in the plasma. This did not occur in a number of experiments. Furthermore, the statistical analysis indicated that the changes in the level of amino acid nitrogen were not significant while those for glutamine were highly significant (see additional discussion in previous paper and table). This problem requires further study.

It is of interest to note that the smaller doses of sodium benzoate (Rabbits nos. 1 and 3) produced the most pronounced rise in blood sugar (total reducing substances). The reason for this effect is not apparent at the present time.

*Summary and conclusions.* 1) Sodium benzoate administered intravenously and orally to rabbits and one human subject was found to depress the level of glutamine in the blood plasma.

2) The intravenous administration of sodium benzoate produced a rise in the total reducing substances in the blood which was highest with the smallest doses of benzoate.

3) The changes in the glutamine level were not related to either the change of the total amino acid level or the level of total reducing substances in the blood.

4) It is suggested that amidation may be either directly or indirectly depressed by the administration of sodium benzoate.

<sup>9</sup> Christensen, H. N., Streicher, J. A., and Elbinger, R. L., *J. Biol. Chem.*, 1948, **172**, 515.

TABLE II.  
Curative Effect of Aureomycin in Polyarthritis of Rats.

	No. of animals*	Incidence of arthritis		Avg arthrogram score	
		Pretreatment, %	4 days after aureomycin, %	Pretreatment	4 days after aureomycin
100 mg aureomycin per kg subcutaneously on 7th and 8th days after infection	20	55	5	1.6	0.15
Untreated controls	20	66	73	1.3	1.35

\* The average body weight in both groups was 75 g.

tration of the drug. The untreated controls showed no measurable level of the antibiotic.

Preliminary clinical trials were made in 4 advanced cases of rheumatoid arthritis which had responded unsatisfactorily to several therapeutic agents, and one early case using 2 g aureomycin daily by mouth for 1 month. Two patients noted an improvement in appetite, and one patient had to discontinue the medication on the third day due to a marked gastrointestinal upset. None of these cases showed any improvement in range of motion of affected joints and 3 of them developed increased pain and swelling while under treatment. One case of Reiter's syndrome responded dramatically, gaining 6 lb during the first week of treatment and losing all joint pain. At the end of one month he showed no

more joint swelling. His blood sedimentation rate (Wintrobe Method) decreased from 40 mm to 12 mm per hour in 2 weeks.

*Summary.* 1. The addition of aureomycin to the diet, subcutaneous administration, and gastric intubation of the antibiotic prevented and cured experimental polyarthritis of rats due to the L<sub>4</sub> strain of pleuropneumonia-like organism.

2. *In vitro* aureomycin prevented growth of the microbe in broth.

3. In preliminary clinical trials, several patients with chronic rheumatoid arthritis who responded unsatisfactorily to several therapeutic agents also failed to respond to aureomycin.

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## 17280. Effect of Incubation on the Cholesterol Partition in Human Serum.\*

KENNETH B. TURNER AND VIRGINIA PRATT.

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Sperry<sup>1</sup> reported that the incubation of blood serum or plasma from normal human subjects resulted in a decrease in the amount of free cholesterol present without a change in the total cholesterol. It was concluded that esterification of some of the free chole-

sterol had taken place and that this had come about through the action of an enzyme, as the process was abolished by heating the serum to 55-60° prior to incubation. If this esterification of cholesterol *in vitro* were the result of enzymatic activity, it seemed possible that the enzyme might be absent or present in reduced amounts in the peripheral blood of patients with an abnormally low cholesterol ester fraction in the serum. This would

\* This investigation was supported by Research Grant No. H39 from the National Heart Institute, U. S. Public Health Service.

<sup>1</sup> Sperry, W. M., *J. Biol. Chem.*, 1935, **111**, 467.

TABLE I.  
Preventive Effect of Aureomycin in Polyarthritis of Rats.

% aureomycin in diet	No. of animals*	Procedure	Incidence of arthritis, %	Avg arthrogram score	Survival, %
0.3	20	Aureomycin added on day of infection and continued for 7 days	0	0	100
0.1	20	Aureomycin added on day following infection and continued for 11 days	0	0	100
0.05	26	Aureomycin added on day of infection and continued for 14 days	15.0	0.07	100
0	10	Intubation dose 50 mg/kg } Aureomycin given by stomach tube (single daily dose) for 2 days fol-	20.0	0.4	100
0	10	20 mg/kg } lowing infection	20.0	0.38	100
0	10	Single dose of aureomycin (100 mg/kg) subcutaneously on day of infection	20.0	0.30	100
0	76	Untreated controls	77.4	2.41	96

\* The average weight of the groups at the beginning was 67 to 75 g.

tered by stomach tube to fasting animals in single daily doses of 50 mg per kg for 2 days, there was an incidence of 20% arthritis with an average arthrogram score of 0.4, while the incidence among the controls was 77.4%, and the average arthrogram score was 2.41, the survival rate being 96%. A single dose of aureomycin, 100 mg per kg, given subcutaneously on the day of infection gave an incidence of 20% and an average arthrogram score of 0.3, compared to a much higher value (2.41) for the controls (Table I).

The curative effect was evaluated by giving aureomycin 100 mg per kg subcutaneously on the 7th and 8th days after the infection was begun and at a time when there was a 55% incidence of arthritis. The crude aureomycin being quite acid caused local necrosis, so further injections were not given. In 4 days the incidence of arthritis had fallen from 55 to 5% in the treated group while it had increased from 66 to 73% in the controls (Table II). At the same time the average arthrogram score for the treated animals had decreased in 4 days from 1.6 to 0.15 while in the controls it had increased from 1.3 to 1.35. At the outset there was a less severe degree of arthritis in

the controls, the drug being tested against a more severe arthritis, yet the outcome was more favorable.

*In vitro*, a concentration of 2  $\mu$ g aureomycin per cc of the broth permitted questionable growth of the microbes in 24 hours and definite growth in 48 hours as estimated by the turbidity of serial dilutions. Using 3  $\mu$ g of aureomycin per cc of broth, no growth was observed.

Estimations of blood levels<sup>†</sup> of aureomycin in the treated rats, according to the method of Brainerd *et al.*,<sup>7</sup> showed that rats receiving 0.1% aureomycin in the Purina dog chow for 3 days had blood levels of 0.62  $\mu$ g per cc, while those eating a diet containing 0.075% aureomycin had less than 0.155  $\mu$ g per cc. Rats receiving 50 mg per kg with a stomach tube once daily for 2 days showed blood levels of 0.62  $\mu$ g of aureomycin per cc when the sample was taken 2 hours after the adminis-

<sup>†</sup> We are indebted to Miss Mirra Scaparoni of the San Francisco Hospital for determination of the aureomycin blood levels.

<sup>7</sup> Brainerd, H. D., Bruyn, H. B., Jr., Meiklejohn, G., and Scaparoni, M., *Proc. Soc. Exp. Biol. and Med.*, 1949, **70**, 318.

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## 17280. Effect of Incubation on the Cholesterol Partition in Human Serum.\*

KENNETH B. TURNER AND VIRGINIA PRATT.

From the Department of Medicine, College of Physicians and Surgeons, Columbia University, and the Presbyterian Hospital, New York City.

Sperry<sup>1</sup> reported that the incubation of blood serum or plasma from normal human subjects resulted in a decrease in the amount of free cholesterol present without a change in the total cholesterol. It was concluded that esterification of some of the free chole-

sterol had taken place and that this had come about through the action of an enzyme, as the process was abolished by heating the serum to 55-60° prior to incubation. If this esterification of cholesterol *in vitro* were the result of enzymatic activity, it seemed possible that the enzyme might be absent or present in reduced amounts in the peripheral blood of patients with an abnormally low cholesterol ester fraction in the serum. This would

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<sup>1</sup>Sperry, W. M., *J. Biol. Chem.*, 1935, 111, 467.

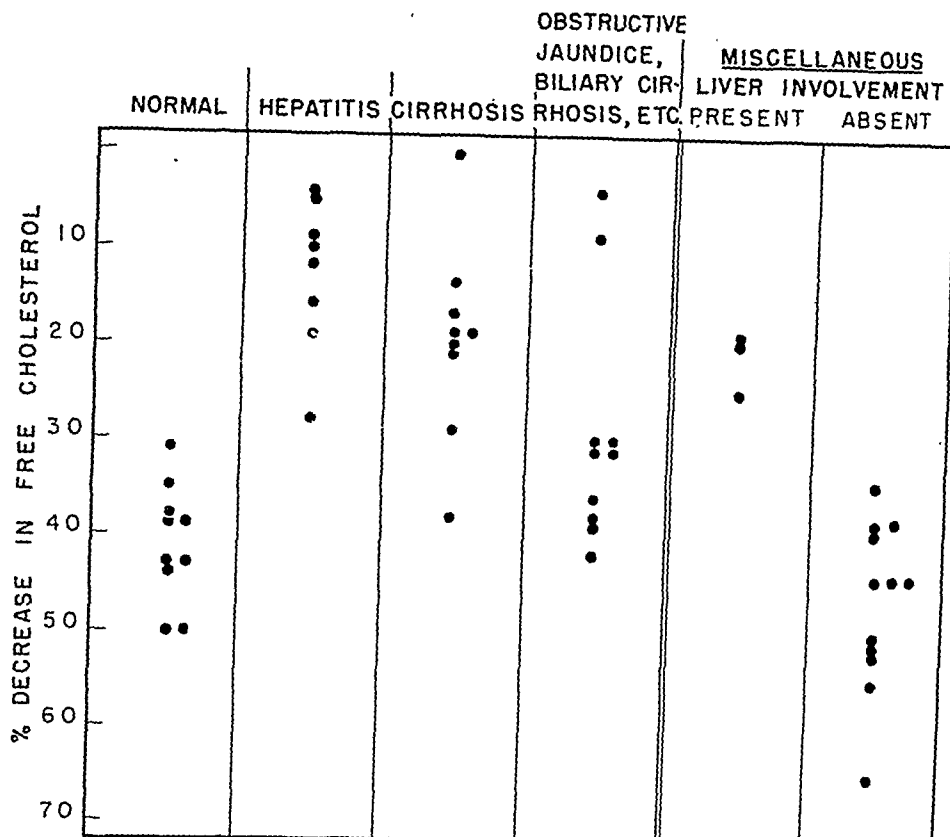


FIG. 1.

The percentage reduction in the amount of free cholesterol in the serum after incubation at 37° for 24 hours. Each dot represents one patient.

occur primarily in liver disease. To test this hypothesis the present investigation was undertaken.

**Experimental.** The effect of incubation on the serum cholesterol partition was studied in 10 normal individuals, 30 patients with liver disease, and 12 patients with a variety of disease conditions but without liver involvement. The concentration of total and free cholesterol in each sample of serum was determined by the method of Schoenheimer and Sperry<sup>2</sup> before and after incubation at 37° for 24 hours. The difference between the total and free cholesterol was assumed to represent the esterified cholesterol. Blood samples were usually obtained in the fasting state. No preservative was added to the serum before incu-

bation. Hemolyzed samples were discarded as Sperry had found that hemolysis inhibits the reaction, and we had confirmed this observation.

The values obtained for the total cholesterol in each specimen before and after incubation were mostly within the limits of error of the method. In other words, the total cholesterol did not change. The decrease in the amount of free cholesterol with incubation varied from none to 69%. The results are summarized in Fig. 1.

In a number of instances the period of incubation was extended to 48 or 72 hours. This seemed to provide little added information even though a further decrease was observed in the amount of free cholesterol, and consequently the 24-hour period was adopted as standard procedure. In one case the decrease

<sup>2</sup> Schoenheimer, R., and Sperry, W. M., *J. Biol. Chem.*, 1934, **106**, 745.

TABLE I.  
Effect of Heating Serum in Preventing a Decrease in Free Cholesterol with Incubation.

		Serum cholesterol				
		Total mg %	Free		Ester	
			mg %	% decrease	mg %	%
Subject 8	Control	268	75		193	72
	Incubated*	267	76	0	191	72
	" †	268	49	35	219	81
Subject 9	Control	180	47		133	74
	Incubated*	181	47	0	134	75
	" †	181	27	43	154	85

\* After heating to 56° for one-half hour.

† Unheated.

in free cholesterol was determined after incubation for 3, 6, 12 and 24 hours. At 3 hours the drop amounted to 13%; at 6 hours it was 18%; at 12 hours, 32% and at 24 hours 42%.

We have confirmed Sperry's observation<sup>1</sup> that heating the serum to 56° before incubation prevents the drop in free cholesterol from taking place, presumably by destroying the enzyme responsible for the reaction. Data on the effect of preliminary heating on the serums of 2 normal subjects are shown in Table I.

**Results.** The decrease in the free cholesterol in the serums of 10 normal individuals ranged from 31% to 50% following incubation. Accordingly, decreases of more than 30% in 24 hours are considered normal, and a decrease of less than 30% is considered abnormal. This agrees with the results of Sperry<sup>1</sup> who, in 30 samples from 22 healthy young adults, obtained a decrease in free cholesterol of 29 to 84% after incubation for 24 to 72 hours.

Among the 30 patients with liver disease there were 8 with acute hepatitis. In all of these the drop in free cholesterol with incubation was less than normal, suggesting that the amount of enzyme present was decreased in this disorder. The decrease in free cholesterol ranged from 4% to 19% in 7, and was 28% in the remaining case. All of the patients in this group showed a low ester cholesterol (25 to 50%) prior to incubation. The serum bilirubin was elevated in all. The cephalin flocculation test was positive in 4,

negative in 4. The alkaline phosphatase exceeded 5 Bodansky units in 5. In 2 patients the serum albumin was less than 4.0 g%. In general, there did not seem to be a close correlation between these other tests and the degree of reduction of free cholesterol on incubation.

In 8 of 9 patients with Laennec's cirrhosis of the liver, the decrease in free cholesterol with incubation was less than the empirical normal, ranging from none to 29%. The proportion of esterified cholesterol before incubation was slightly below normal (60 to 69%) in 7, and low (18%) in one. The serum bilirubin was increased in 7. The cephalin flocculation test was positive and the serum albumin reduced also in 7 cases. The alkaline phosphatase was between 5 and 6 Bodansky units in 3; less than 5 units in 5. The ninth patient in the group was admitted to the hospital because of bleeding esophageal varices, and was considered to have inactive cirrhosis. The serum of this patient showed a normal drop of 38% in free cholesterol after incubation, and all other chemical tests were normal as well.

In another group were 10 patients with obstructive jaundice, biliary cirrhosis, or extensive metastatic carcinoma of the liver. A characteristic of the group was an elevated alkaline phosphatase exceeding 10 Bodansky units. A reduction in free cholesterol following incubation that was interpreted as being within the normal or low normal range occurred in 8 of the 10 cases. The proportion of esterified cholesterol before incubation was less than 70% in 9 of these 10 cases. The



serum bilirubin was elevated in 7, the cephalin flocculation test was positive in one, abnormal A/G ratios were present in 2.

In a final group among the examples of liver disease there were 3 patients with miscellaneous disorders. One patient had disseminated lupus with ascites, a marked reduction in serum albumin, and a positive cephalin flocculation. Another was admitted in severe congestive heart failure with clinical jaundice and was found to have an elevated serum bilirubin, positive cephalin flocculation, and 50% bromsulfalein retention. The third patient showed extreme involvement of the liver by Hodgkin's disease at autopsy. The decrease in free cholesterol following incubation of serum from these 3 patients was interpreted as less than normal in all—19%, 25% and 20%.

As a further control, 12 patients with various diseases but without demonstrable liver involvement were studied. The group included 4 cases of anemia, 2 cases of fever of unknown origin, and single examples of optic neuritis, leukemia, chronic glomerulonephritis, diabetes, ichthyosis, and psoriasis. In all of these patients the reduction of the free cholesterol with incubation was considered normal. The decrease ranged from 35% to 69%. In 8 of these cases the proportion of esterified cholesterol before incubation was above 70%; in 4 it was between 58% and 69%.

Two or 3 determinations of the reduction of free cholesterol have been made at varying intervals on the serums of each of 6 patients. In a diabetic the decrease was constant on 3 occasions within 8 days. In two patients with infectious hepatitis who showed a decreased reduction in the amount of free cholesterol originally, the test returned to within normal limits with clinical improvement; in a third patient with this disease there was a slight progressive decline in the reduction of free cholesterol until the death of the patient. A patient with cirrhosis, who at first had an abnormally slight decrease in the amount of free cholesterol with incubation, later showed a reduction considered within the normal range. This was accompanied by very little change in clinical condition and

chemically only by a rise in serum albumin from 2.7 g to 3.3 g. A patient with heart failure and clinical jaundice, who at first had a reduction of 25% in free cholesterol with incubation showed a normal reduction of 46% after clinical improvement and disappearance of the jaundice.

**Discussion.** The mechanism of the reaction described is not clear. An enzyme appears to be involved in the reduction in the amount of free cholesterol in the serum following incubation. This enzyme effect is decreased when the liver parenchyma is involved as in hepatitis and cirrhosis, and also occasionally in obstructive jaundice. Work is in progress in an attempt to elucidate the mechanism and to establish the clinical significance of the reaction here described.

The decrease in free cholesterol with incubation does not parallel the rise in serum bilirubin. Sperry<sup>3</sup> found that the addition of bile salts to the serum *in vitro* inhibited the reaction, but we have found a normal reduction in free cholesterol in the presence of clinical jaundice and a decreased reduction without an increase in the serum bilirubin. There is certainly no correlation between this test and the cephalin flocculation test, alkaline phosphatase or serum albumin. There seems to be a somewhat better correlation with the proportion of esterified cholesterol in the serum before incubation, but even in this there are striking exceptions.

While the change in free cholesterol has been given in percentages, it should be pointed out that it may eventually prove desirable to express this in milligrams per cent. Decision on this point must await further information from work now in progress on the nature of the enzyme and its action.

**Summary.** Incubation of normal human serum and that of patients with disease not involving the liver results in a drop in free cholesterol of more than 30% without change in the total cholesterol. The reaction is probably due to an enzyme, as the decrease does not occur when the serum is heated to 56° before incubation.

<sup>3</sup> Sperry, W. M., and Stoyanoff, V. A., *J. Biol. Chem.*, 1937, **117**, 525.

In this small series, the decrease in free cholesterol with incubation was usually less than normal with disease of the liver parenchymia and occasionally with extra-hepatic obstructive jaundice. The decrease in free cholesterol apparently did not parallel

changes in serum bilirubin, alkaline phosphatase, serum albumin, or the cephalin flocculation test. There appeared to be some correlation with the percentage of cholesterol esters in the control serum.

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## 17281. Effect of Antihistamine on the Localization of Trypan Blue in Xylene Treated Areas of Skin.

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Some data and experimental observations have suggested that histamine may play a fundamental role in the development of local areas of inflammation.<sup>1-3</sup> In support of this, it has been shown that trypan blue following an intravenous injection localizes and concentrates in areas of skin previously injected with histamine.<sup>4</sup> The effects of antihistamine drugs on capillary permeability have been reviewed recently by Last and Loew.<sup>5</sup> These investigators found that the localization of trypan blue in areas of skin previously injected with different chemical and biological preparations was not modified by the intravenous injection of the antihistamine preparation "Benadryl".<sup>5</sup>

The present experiments were performed to study the effect of two of the more recent antihistamine preparations on the hyperemia and the localization and concentration of trypan blue in areas of skin treated with xylene.

**Methods and materials.** Fifteen rabbits were used. Their hair was carefully removed 24 to 48 hours preceding the time of the

experiment. Two antihistamine preparations were used: Pyrrolidineethyl-phenothiazine hydrochloride (Pyrrolazote\*); and, N,N-Dimethylly-N' (alpha-pyridyl)-N' (alpha-thenyl)-ethylenediamine hydrochloride (Thenylene†). A solution of each containing 10 mg per cc was made in physiologic sodium chloride. The injections of Pyrrolazote were made intravenously, while Thenylene was injected both intravenously and intraperitoneally. Ten cc of a 0.2% solution of trypan blue was injected intravenously. Xylene was carefully applied with a cotton applicator to local areas of skin at intervals varying from one to 140 minutes during the time that the rabbits were under the influence of the antihistamine preparations and before the dye was injected intravenously. The xylene treated areas were carefully observed during the development of hyperemia and during the time of the localization and concentration of the dye. These observations extended over a period of 2 hours.

In 8 rabbits 0.2 cc of the antihistamine preparations was injected intradermally from immediately to 60 minutes preceding the time of the intravenous injection of trypan blue. An equal volume of both a physiologic sodium chloride solution and distilled water was used as controls.

<sup>1</sup> Lewis, Sir Thomas, London, Shaw and Shaw, 1927.

<sup>2</sup> Findlay, G. M., *J. Path. and Bact.*, 1928, **31**, 633.

<sup>3</sup> Mayer, R. L., *Ann. Allergy*, 1947, **5**, 113.

<sup>4</sup> Rigdon, R. H., *J. Lab. and Clin. Med.*, 1942, **27**, 1554.

<sup>5</sup> Last, M. R., and Loew, E. R., *J. Pharm. and Exp. Therap.*, 1947, **80**, 81.

\* Obtained from the Upjohn Company, Kalamazoo, Mich.

† Obtained from the Abbot Research Laboratories, Chicago, Ill.

*Experimental.* Four rabbits were given Pyrrolazote intravenously. One received 23.31 mg/kg in 5 injections during a period of 85 minutes; a second, 24.21 mg/kg in 4 injections during a period of 48 minutes; a third, 15.54 mg/kg in 3 injections during a period of 137 minutes and the fourth, 16.5 mg/kg in 2 injections during a period of 16 minutes.

The areas of skin where xylene was applied became hyperemic within a minute and progressively increased in intensity for approximately 5 minutes, after which the reaction remained more or less constant for several hours then regressed. The rate of development and the intensity of the hyperemia that occurred following the application of xylene did not vary from that in rabbits similarly treated, but not given any antihistamine as previously reported.<sup>6</sup> Trypan blue likewise localized and concentrated in these xylene treated areas in a manner similar to that observed in rabbits not given any antihistamine.

All the xylene treated areas of skin were hyperemic. Trypan blue, however, localized and concentrated first in the last 2 areas where xylene was applied the shortest interval before the dye was given.

Three rabbits were given Thenylene both intravenously and intraperitoneally. One was given 28.11 mg/kg in 6 injections over a period of 37 minutes, another 16.64 mg/kg in 4 injections over a period of 35 minutes, and one, 17.57 mg/kg in 4 injections during a period of 40 minutes.

The areas of skin where xylene was applied were identical in these rabbits with those observed in animals given the Pyrrolazote and trypan blue. These 3 rabbits showed toxic symptoms produced by this antihistamine preparation during the time of the experiment.

Trypan blue localized and concentrated in each skin area of the 8 rabbits where both antihistamine drugs were injected intradermally. In 2 rabbits injected with Thenylene there was less dye after 30 minutes in the areas injected 35 minutes before the intravenous injection than in the areas injected 15 minutes and immediately preceding the intravenous injection of the dye. Trypan blue did not concentrate in any of the areas of skin pre-

viously injected with physiologic sodium chloride. Twenty minutes following the intravenous injection of trypan blue there was a small area at the site of the injection of the antihistamine preparation that was pale in color and did not stain blue. There was a zone of dye around this anemic area and, peripheral to it, there was a zone of edema which did not stain any deeper with trypan blue than surrounding normal skin. There were no significant changes in the manner of localization of trypan blue in the injected areas of skin after the first 30 minutes of the experiment.

*Discussion.* In these observations the two antihistamine preparations, Thenylene and Pyrrolazote, in the concentrations used apparently do not effect the development of hyperemia and the localization and concentration of trypan blue in xylene treated areas of skin. In previous studies it has been emphasized that the localization and concentration of trypan blue in areas of inflammation are not determined by the presence of hyperemia.<sup>6</sup> In support of this observation, Last and Loew have found that acetyl-B-methyl-choline (Mecholyl), although producing vasodilation, did not cause a trypan blue reaction.<sup>7</sup> Can it be that a local area of skin treated with xylene or injected with horse serum stains blue primarily as a result of a variation in the absorptive ability of the tissue cells, and not because there is only a change in the permeability of the capillaries? The vital staining of cells has been regarded by some as due to absorption of dye molecules by cell granules.<sup>7</sup>

According to Last and Loew,<sup>5</sup> appropriate concentrations of intradermally injected horse serum, tetracaine, codeine and heparin cause positive trypan blue reactions which are not modified by Benadryl. Arginin, which is a specific antagonist against some of the effects of histamine, does not prevent the production of a trypan blue reaction by either histamine or agents which liberate histamine.<sup>8</sup> The above observations are different from those

<sup>6</sup> Rigdon, R. H., *Arch. Surg.*, 1940, **41**, 101.

<sup>7</sup> Nagao, K., *J. Inf. Dis.*, 1921, **28**, 294.

<sup>8</sup> Rocha e Silva, M., and Dragstedt, C. A., *J. Pharm. and Exp. Therap.*, 1941, **73**, 405.

of Mayer who found that "pyribenzamine, a substance exhibiting strong and specific antihistamine properties, exerts a definite activity in experimental dermatitis".<sup>3</sup>

The localization and concentration of trypan blue following an intravenous injection, in areas of skin injected intradermally with antihistamine emphasizes the fact that substances other than histamine may cause a localization of this dye.

**Summary** The hyperemia that follows the

local application of xylene apparently is not modified by the intravenous and intraperitoneal injections of the antihistamine preparations, Pyrrolazote and Thenylene. Likewise, the localization and concentration of trypan blue in the xylene treated areas of skin are not affected by these preparations of antihistamine. Trypan blue also localizes and concentrates in areas of skin injected intradermally with these preparations of antihistamine.

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## 17282. Studies on Elimination of Penicillin G in Dogs.\*

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From the Department of Medicine, University of Chicago.

Rammelkamp and Keefer<sup>1</sup> found that only about 60% of intravenously administered penicillin could be recovered from the urine of man. This finding has been confirmed by later reports, although the recovered amounts vary from 40 to 99%.<sup>2</sup>

This paper deals with the mechanism of the elimination of penicillin in bilaterally nephrectomized dogs.

**Methods.** Healthy mongrel dogs weighing from about 6 to 12 kg were used. Crystalline penicillin G§ was injected intravenously in all experiments; the blood samples were obtained from another vein and the serum penicillin

assayed against *Staphylococcus* 209 by a serial dilution technic.

**Diffusion of Penicillin from Blood to Tissues. Results.** A dog which had been bilaterally nephrectomized and the cystic and common bile ducts ligated was given an intravenous injection of 25,000 u/kg of crystalline penicillin G. The penicillin concentration decreased rapidly during the first hour but very slowly thereafter (Fig. 1). It is assumed that this initial decrease was due to

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† Douglas Smith Foundation Fellow in Medicine.

‡ Research Fellow, U. S. Public Health Service.

<sup>1</sup> Rammelkamp, C. H., and Keefer, C. S., *J. Clin. Invest.*, 1943, 22, 425.

<sup>2</sup> Herrell, W. E., *Penicillin and Other Antibiotic Agents*, W. B. Saunders Company, 1945.

§ The crystalline penicillin was supplied by Abbott Laboratories, Commercial Solvents Corporation, Lederle Laboratories, and Schenley Laboratories, Inc.

The diffusion of penicillin from blood to tissues

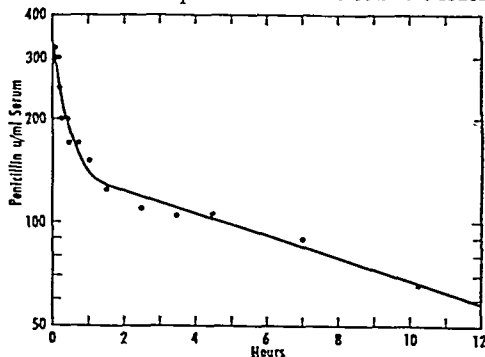


FIG. 1.

The serum penicillin concentration as a function of the time after a single intravenous injection. The inactivation has been partially blocked by bilateral nephrectomy and ligation of the cystic and common bile ducts.

TABLE I.

Dose units/kg	$c_{\infty}$ units/ml	$c_{\infty}$ units/ml dose units/g of dog
85,000	400	4.7
80,000	250	3.1
30,000	130	4.3
25,000	90	3.6
25,000	70	2.8
25,000	80	3.2
25,000	100	4.0
25,000	85	3.4
25,000	100	4.0
25,000	110	4.4
25,000	80	3.2
25,000	100	4.0
25,000	140	5.6
14,000	60	4.3
14,000	60	4.3
7,000	20	2.9
Average — 3.9		
Maximum—5.6		
Minimum—2.8		

The serum concentration ( $c_{\infty}$ ) of penicillin after diffusion equilibrium has been established with the tissues. Values are corrected for any elimination or inactivation of penicillin taking place.

diffusion of penicillin from the blood stream into the tissues.

If there were no further elimination of penicillin from the blood, the concentration would decrease to a constant value which would be reached when diffusion equilibrium was established. This concentration may be found by drawing the straight line representing the inactivation back to intersection with the axis of the ordinate which, in this case, was 143 u/ml of serum.

The equilibrium concentration was determined in a number of experiments and collected in Table I. In the last column of this table are the ratios between  $c_{\infty}$  (serum concentration of penicillin at equilibrium) and the dose of penicillin per gram of dog. In 16 experiments this ratio was found to be larger than unity. This means that the penicillin did not distribute equally throughout the tissues of the dog but was present in a higher concentration in the plasma than in the remainder of the dog. The average ratio was about 4 times as great when diffusion equilibrium was established. This relationship can be expressed as:

Serum penicillin concentration = 4 x amount of penicillin per gram body weight. The formula has been established for the

blood concentrations between 20 and 400 u/ml and may not be valid for smaller concentrations.

*The Renal and Extra-Renal Elimination.* Blood serum concentrations of penicillin were determined at intervals after a single intravenous injection in a number of normal dogs. When allowance was made for the diffusion equilibrium to be established, it was found that the logarithm of the concentration decreased as a linear function of time (First order reaction). This relationship was found to be valid from the initial concentration of about 100 u/ml to 1 u/ml. A curve demonstrating this relationship is shown in Fig. 2.

Bilateral nephrectomy was performed in 3 dogs which were then given a single intravenous injection of penicillin. These dogs were still able to eliminate penicillin from the blood stream, although at a considerably lower rate (Fig. 2). The average rate of elimination was found to be  $\frac{1}{4}$  as rapid in the nephrectomized as in the normal dogs. This indicates that approximately three-fourths of the penicillin was eliminated through the kidney and one-fourth elsewhere. From the variable velocities obtained in normal and nephrectomized dogs, a considerable variation was to be expected in the fraction of penicillin inactivated extra-renally.

The rate of elimination of penicillin after various surgical procedures

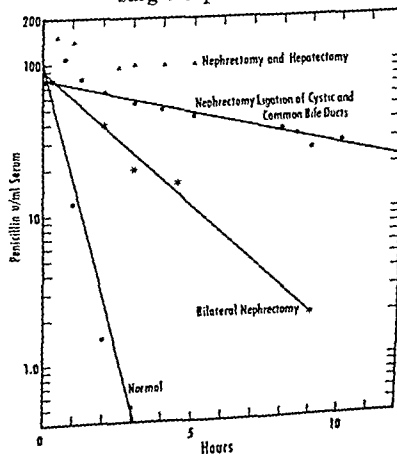


FIG. 2.

All dogs were given 25,000 units of penicillin per kilogram in a single intravenous injection.

*Site of the Extra-Renal Elimination.* The rate of elimination was not significantly altered by the removal of the intestine from the ligament of Treitz to within an inch of the anus; nor by the removal of the stomach and ligation of the common bile duct. This showed that in these dogs the intestinal tract was not the primary site of the extra-renal elimination, as suggested by the findings of Reid.<sup>3</sup>

Following complete evisceration the serum penicillin concentration remained constant, within the limits of experimental error; thus the site of the elimination was somewhere in the viscera.

A two stage hepatectomy and bilateral nephrectomy were performed in a dog. Here too, no inactivation could be shown after the equilibration of penicillin between the blood stream and the tissues. In another dog, both kidneys and all the viscera except the liver were removed. The hepatic artery was left intact by dissecting away the hepaticoduodenal ligament. The portal vein and the common bile duct were ligated and the gall bladder left in connection with the biliary system. This dog eliminated penicillin at a rate very similar to the dogs in which only bilateral nephrectomy was done. It could be concluded that the liver was responsible for the extra-renal elimination of penicillin by these dogs. The velocity constants indicating the relative decrease in penicillin concentration, or total amount of penicillin per hour, are shown in Table II. The velocity constants were found from the curves by the formula:

$$K = \frac{\ln (C_{t_1}/C_{t_2})}{t_1 - t_2}$$

*Mechanism of Hepatic Elimination of Penicillin.* In 2 dogs the kidneys were removed, the cystic and common bile ducts were ligated twice and cut. Similar curves were found in both dogs; one curve is shown in Fig. 3. It was noted that a certain elimination of penicillin seemed to occur during the first 10 hours; however, the rate of elimination decreased after this to a very low value. Thus,

<sup>3</sup> Reid, R. D., Felton, L. C., and Pittroff, M. A., *Proc. Soc. Exp. Biol. and Med.*, 1946, **63**, 438.

TABLE II.  
The Velocity Constant Indicating the Relative Decrease in Penicillin Concentration in the Serum After Various Surgical Procedures.

Procedure	K 1/hr	Avg
Normal dogs	1.85	1.70
	1.90	
	2.40	
	1.90	
	1.25	
	1.15	
Nephrectomized	1.45	0.41
	0.46	
	0.38	
Nephrectomized and the intestine removed from the ligament of Treitz to within one inch of the anus	0.40	0.34
	0.33	
Nephrectomized, bile duct ligated and complete gastrectomy	0.35	
Eviscerated, except for the liver	0.40	
Complete evisceration	0.53	
Hepatectomy, nephrectomy	0.0	
Cystic and bile ducts ligated; nephrectomized	0.07	
Bile duct cannulated	0.31	
—excreted in bile—	0.25	
Inactivation in bile at —log eH+ 8.5, 40°C	0.10	

penicillin was still present in appreciable amounts 70 hours after the intravenous injection. The final rate of elimination was only 4% of the normal rate. This finding seemed to indicate that the penicillin was excreted in the bile rather than destroyed by the liver. If so, the initial drop in serum concentration might be due to excretion of bile into the biliary system to the point of distention. However, it still appeared possible that destruction might have taken place in the liver, but that liver function was impaired by the biliary obstruction.

In another nephrectomized dog, the common bile duct was cannulated and the secreted bile collected hourly after an intravenous injection of penicillin. Very high concentrations of penicillin were found in the bile samples. The total amount recovered in the bile was 80% of the injected penicillin. It could be

concluded that, at least in this dog, the main part of the extra-renal elimination took place as excretion in the bile.

**Inactivation by bile in vitro.** A sample of dog's bile (pH 8.29) obtained from a common duct fistula, was mixed with penicillin and incubated at 37°C. A rather slow inactivation

The slow elimination after bilateral nephrectomy and ligation of cystic and common bile ducts

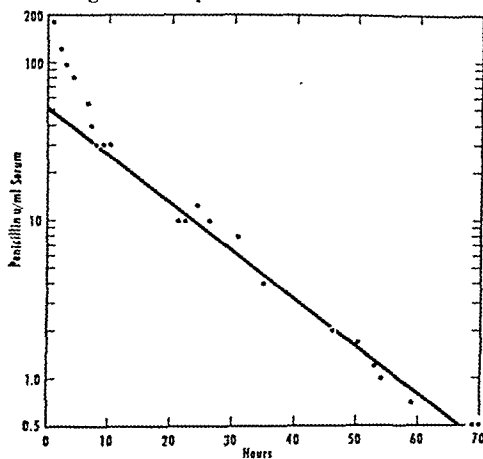


Fig. 3.

The elimination during the first 10 hours may be due to excretion of penicillin-containing bile into the biliary system to the point of distention.

The slower inactivation throughout the 70 hours of the experiment can be explained by the destruction of penicillin due to the alkalinity of the bile.

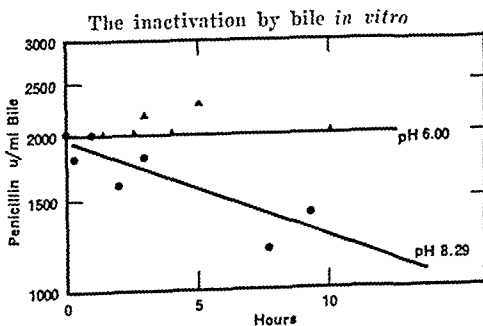


Fig. 4.

The lines show the inactivation, at the given pH values as calculated from the data of Brodersen,<sup>4</sup> in good agreement with the experimental points.

The two samples of bile were obtained from the gall bladder (pH 6.00) and from a common duct fistula (pH 8.29).

The pH values were determined electrometrically by comparison with 0.01 M HCl + 0.5 M NaCl (pH 2.00). The hydrogen ion concentrations were found to be independent of temperature in the range of 20 to 40°C.

Bile and serum penicillin concentration after an intravenous injection of penicillin in a nephrectomized dog

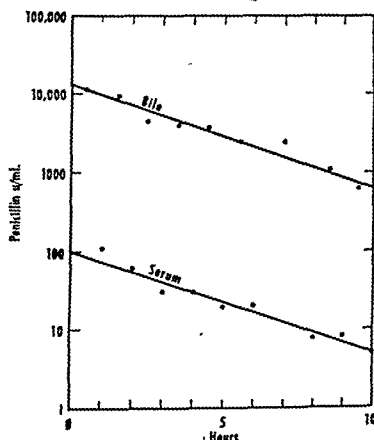


Fig. 5.

The bile is seen to contain about 130 times as much penicillin per milliliter as the serum, independent of the serum concentration.

tion was found to take place. Another sample drawn from the gall bladder (pH 6.00) did not inactivate penicillin at a demonstrable rate. This suggested that the inactivation was due to the alkalinity of the fistula bile. The rate of inactivation to be expected in this case was calculated<sup>4</sup> and found to be consistent with the experimental values (Fig. 4). The rate of inactivation in the bile can thus be calculated at known values of temperature and hydrogen ion concentration on the basis of the known destruction rate by alkali.

**Relationship of Penicillin Concentrations in Serum and Bile.** In Fig. 5 are plotted the concentrations of penicillin in cannulated bile and in blood serum taken simultaneously from a nephrectomized dog. There was a constant ratio between the two concentrations, the bile containing 130 times as much penicillin per ml as the serum at serum concentrations ranging from 100 to 5 units/ml. This ratio was found to vary considerably. In 7 experiments carried out in two dogs, values ranging from 10 to 330 were obtained. From the figures in Table III it is seen that the high ratios generally were found at high pH values in the bile and the low ratios at the low pH values.

<sup>4</sup> Brodersen, R., *Trans. Farad Soc.*, 1947, **43**, 351.

TABLE III.  
Correlation Between Bile pH and the Bile Penicillin-serum Penicillin Ratio.

pH of bile	Penicillin concentration of bile
	Penicillin concentration of serum
8.65	330
8.50	130
8.48	270
8.29	10
8.20	16
8.04	11
7.93	11

The pH values were determined at the same time and from these the spontaneous inactivation calculated as shown by the curve. The difference between this curve and the points demonstrate the resorption from the gall bladder. Within the 4 hours of the experiment the resorbed amount is seen to be rather small. That a certain resorption does take place was shown by the fact that penicillin was present in the urine of the dog.

*Summary.* Bilaterally nephrectomized dogs eliminated penicillin G at a considerable rate from their blood stream after an intravenous injection. This elimination took place at essentially the same rate when either the intestines were removed, or when the common bile duct was ligated and the stomach removed. But no inactivation could be demonstrated after complete evisceration or hepatectomy in nephrectomized animals. A major portion of the injected penicillin was excreted in the bile. Appreciable inactivation was found in the bile, especially in the case of a strongly alkaline bile.

The remaining portion of penicillin may have gone either to the gall bladder, where only a slow but significant resorption took place, or to the intestine where it was partially resorbed and partially inactivated.<sup>5</sup>

Caution must be exercised in drawing generalized conclusions from these results. Since the nephrectomized dogs do not eat, it seems possible that normal dogs might inactivate certain amounts of penicillin in their intestine because of a greater secretion of digestive juices.

Resorption and inactivation of penicillin in the gall bladder

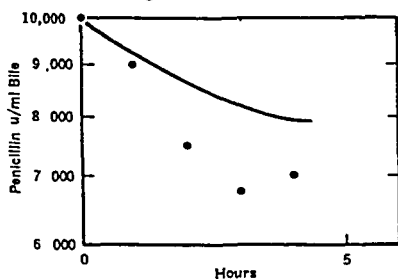


FIG. 6.

The curve indicates the calculated inactivation. The experimental points are seen to deviate relatively little from this curve, indicating that only a slow resorption of penicillin takes place from the gall bladder.

<sup>5</sup> Seeberg, V. P., Illg, P. I., Brown, D. J., *J. Am. Pharm. Assn.*, 1946, **35**, 280.



concluded that, at least in this dog, the main part of the extra-renal elimination took place as excretion in the bile.

**Inactivation by bile *in vitro*.** A sample of dog's bile (pH 8.29) obtained from a common duct fistula, was mixed with penicillin and incubated at 37°C. A rather slow inactivation

The slow elimination after bilateral nephrectomy and ligation of cystic and common bile ducts

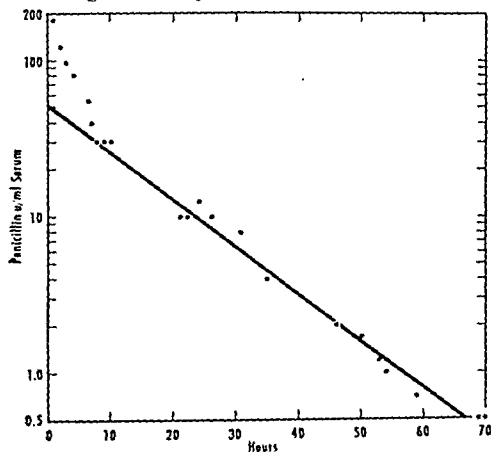


FIG. 3.

The elimination during the first 10 hours may be due to excretion of penicillin-containing bile into the biliary system to the point of distention.

The slower inactivation throughout the 70 hours of the experiment can be explained by the destruction of penicillin due to the alkalinity of the bile.

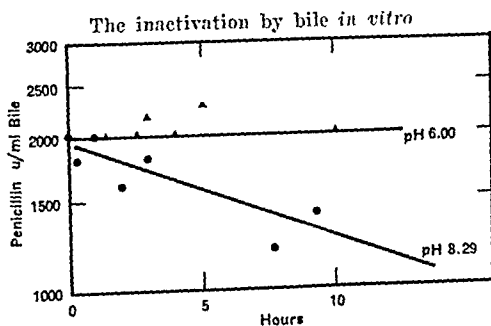


FIG. 4.

The lines show the inactivation, at the given pH values as calculated from the data of Brodersen,<sup>4</sup> in good agreement with the experimental points.

The two samples of bile were obtained from the gall bladder (pH 6.00) and from a common duct fistula (pH 8.29).

The pH values were determined electrometrically by comparison with 0.01 M HCl + 0.5 M NaCl (pH 2.00). The hydrogen ion concentrations were found to be independent of temperature in the range of 20 to 40°C.

Bile and serum penicillin concentration after an intravenous injection of penicillin in a nephrectomized dog

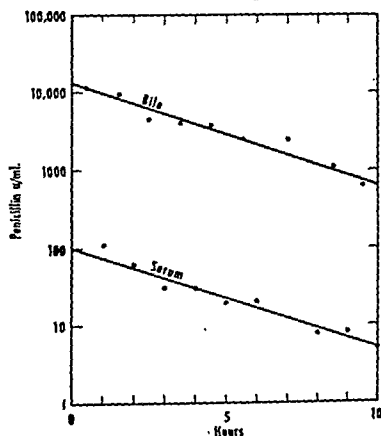


FIG. 5.

The bile is seen to contain about 130 times as much penicillin per milliliter as the serum, independent of the serum concentration.

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<sup>4</sup> Brodersen, R., *Trans. Farad. Soc.*, 1947, **43**, 351.

TABLE II.  
Agglutination\* in Type A (RE) Serum after Absorption with Homologous and Heterologous Cells.

Cells	Unabsorbed serum	Serum absorbed with:									
		732	2526	4189	BT	RE	DU	BE	IIA	1523	L2
732	+									+	+
2526	+									+	+
4189	+									+	+
BT	+									+	+
RE	+									+	+
DU	+									+	+
BE	+									+	+
IIA	+									+	+
1523	+									+	+
L2	+									+	+
LE	+									+	+

\* Microscopic slide technic; serum dilution 1:20.

Agglutinations were performed by the serial dilution tube test and by a microscopic slide technic using serum dilutions of 1:20.

After twelve weekly series of injections agglutinating titers of 1:320 were obtained against strains DU, RE, and 1523. Strain LE produced a titer of 1:40.\* The other strains with the exception of RO and ATCC 732 had developed low titers (1:10 to 1:40) at the end of 12 weeks.

Agglutinin absorptions were conducted by the method of Krumwiede, Cooper and Provost.<sup>8</sup>

**Results.** From the data presented in Table I, it is evident that serologic differences exist among the strains employed. For convenience, the 3 varieties have been designated as Types A, B, and C. With the exception of anti-DU serum, it was necessary to absorb all serum with heterologous cells to prevent cross-reactions. The anti-DU serum agglutinated all Type A strains to a titer of 1:320, but failed to agglutinate Types B and C. Table II presents the results of slide agglutinations in anti-RE (Type A) serum following absorption with homologous and heterologous cells. These data were confirmed by similar absorptions of Type B (1523) and Type C (LE) sera.

**Discussion.** There appears to be a difference in the immunogenic properties of different strains of *C. neoformans*. Although the rabbits used in the present investigation received equal numbers of killed cells, high titers were produced against only 3 strains. This variation may account for the failure of some authors to elicit antibody formation.

Although it is obvious that there are differences in the antigenic structure of the strains used here, no conclusions can be drawn regarding the location of the antigens responsible. It seems possible that the capsular carbohydrate may be important since Neil and his associates<sup>5</sup> have reported a polysaccharide

\* Although this serum is of considerably lower titer than the others, it was included in the reciprocal agglutination tests because strain LE appeared to be heterologous to all other strains used.

<sup>8</sup> Krumwiede, C., Cooper, G., and Provost, C. J., *J. Immunol.*, 1925, **10**, 55.

# 17283. An Immunologic Comparison of Twelve Strains of *Cryptococcus neoformans* (*Torula histolytica*).

E. EDWARD EVANS. (Introduced by John F. Kessel.)

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The results of various authors concerning the immunogenic properties of *Cryptococcus neoformans* have not been in agreement.<sup>1-7</sup> Some investigators have been unable to demonstrate antibody formation in animals<sup>4,7</sup> although others have reported agglutinin titers ranging from 1:9<sup>5</sup> to 1:280.<sup>3</sup> Benham<sup>1</sup> obtained serum with an agglutinin titer of 1:160 against pathogenic strains by injecting capsule free cells into rabbits. Working with pathogenic and non-pathogenic strains she has divided the genus *Cryptococcus* into 4 groups on the basis of serologic and morphologic characteristics. The present investiga-

tion is concerned only with strains isolated from human infections (Benham Group III).

**Methods.** Rabbits were immunized with encapsulated, formalin-killed cells of *C. neoformans*. Intravenous injections were made on three consecutive days of each week. Nine strains of *C. neoformans* were employed for immunization: strains BT, RE, DU, 1523, RO, L2, and LE from the Los Angeles County General Hospital and strains 732 and 2526 from the American Type Culture Collection. Three additional strains were included in the agglutination tests: HA,<sup>†</sup> BE,<sup>‡</sup> and ATCC 4189.

TABLE I.  
Agglutination\* in Serum Absorbed with Cells of Heterologous Type.

Type	Cells	Serum		
		Type A (RE) <sup>†</sup>	Type B (1523) <sup>‡</sup>	Type C (LE) <sup>§</sup>
A	732	++++	—	—
	2526	++	—	—
	4189	++	—	—
	BT	++++	—	—
	RE	++++	—	—
	DU	++++	—	—
	BE	++++	—	—
	HA	++++	—	—
B	1523	—	+++	—
	L2	—	+++	—
	RO	—	++	—
C	LE	—	—	++++

\* Microscopic slide technic; serum dilution 1:20.

† Absorbed with strain L2. Similar results were obtained when other Type B strains were used for absorption. (See Table II).

‡ Absorbed with strain RE. Similar results were obtained when other Type A strains were used for absorption.

§ Absorbed with Type B cells.

<sup>1</sup> Benham, R. W., *J. Inf. Dis.*, 1935, **57**, 255.

<sup>2</sup> Drake, C. H., *Proc. Soc. Am. Bact.*, 1948, **1**, 57.

<sup>3</sup> Hoff, C. L., *J. Lab. and Clin. Med.*, 1942, **27**, 751.

<sup>4</sup> Kligman, A. M., *J. Immunol.*, 1947, **57**, 395.

<sup>5</sup> Neil, J. M., Castillo, C. G., Smith, R. H., and Kapros, C. E., *J. Exp. Med.*, 1949, **89**, 93.

<sup>6</sup> Rappaport, B. Z., and Kaplan, B., *Arch. Path. and Lab. Med.*, 1926, **1**, 720.

<sup>7</sup> Sheppe, W. M., *Am. J. Med. Sci.*, 1924, **167**, 91.

† Obtained from Dr. M. Marples, Otago University Medical School, New Zealand.

‡ From the Los Angeles County General Hospital.

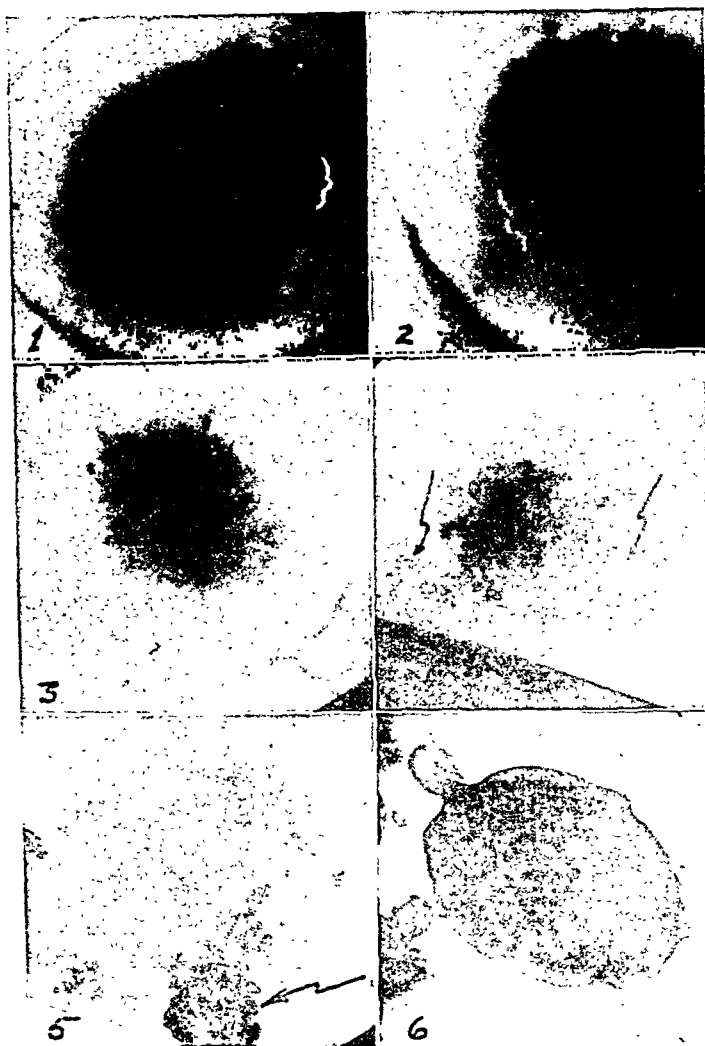


PLATE I.

Electron micrographs of osmosis-hemolyzed chick embryo erythrocytes.  $\times 4000$ .  
 Fig. 1, 2, 3, 4 and 5: Unfixed and unstained. Salt residues indicated at arrows.  
 Fig. 6: Osmic acid fixed where fixation seems to be excessive.

lack of nucleoli in their nuclei; and by the high degree of permeability to electrons of the cytoplasm over the nucleus. They are easily differentiated further from isolated nuclei by their outlines, for where those of the erythrocytes are sharp and well-distinguished, those of the nuclei are diffuse and indistinct.

The nucleus appears clearly in these erythrocytes, except where they have been osmic acid or silver nitrate fixed when detail over

almost the whole body of the cell is lost. The nucleus has normal oval appearance in the unfixed, unstained cells and is centric, diffuse-edged and reticular in structure. It shrinks and deforms when it has been formalin-fixed and no structure is seen in it.

Several degrees of hemolysis were apparently provoked by the hypotonic solutions employed in the preparation of the material. Thus, there are different degrees of opacity of the cytoplasm to electrons in each of Fig. 1, 4

from *Cryptococcus* which was serologically active up to a dilution of 1:2,000,000.

**Summary.** 1. Rabbits were immunized with 9 strains of *Cryptococcus neoformans*. After 12 weekly series of injections, 3 strains produced agglutinin titers of 1:320, 4 strains gave titers ranging from 1:10 to 1:40 and

2 strains failed to produce antibodies.

2. On the basis of reciprocal agglutinin absorptions, 3 serologic types of the organism are described. These have been designated as Types A, B, and C.

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## 17284. Electron Microscopy Study of Chick Embryo Erythrocytes.

JUAN J. ANGULO AND JOHN H. L. WATSON. (Introduced by O. H. Gaebler.)

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A few electron micrographs of erythrocytes are found in the literature.<sup>1-7</sup> Erythrocytes with cytoplasm made transparent to electrons apparently by the hypotonic solutions which were used in specimen preparation were discovered accidentally in preparations of isolated liver cell nuclei which were being examined in an electron microscope.

**Materials and methods.** Nuclei of liver cells from healthy, 16-day-old chick embryos were isolated by the differential centrifugation technic of Dounce<sup>8</sup> as modified by Hoerr,<sup>9</sup> and a portion of the final sediment was resuspended in distilled water for immediate deposition upon electron microscope specimen screens. Three other portions were resuspended for ten minutes in 10% formalin, in 2% osmic acid and in 1% silver nitrate, respectively, and specimens of each were prepared as before. The proportion of reagent volume to specimen volume was about 10:1 and

seemed excessive, but smaller proportions of reagent and less time for reagent action gave bad results also. Some of the prepared screens which were formalin-fixed only were stained with Harris hematoxylin for 5 minutes; others with 1% safranin for one minute and still others with 1% methyl green for one minute and were washed with distilled water to remove the excess stain. Wet preparations were examined with the ordinary light microscope for control purposes.

Micrographs were taken employing a biased electron gun and an objective aperture with relatively low plate magnifications between 3,000 and 4,000 times. Crystalline residues, which are recognizable in electron micrographs were often observed, and examples of some are indicated at the arrows in Fig. 4, 5 and 12. No effects of electron bombardment were noted upon the morphology of the cells, except that they shrunk when focussed beams were directed upon them, but none of the fields reproduced here have been subject to focussed beams.

**Observations and discussion.** Unfixed, unstained erythrocytes are identified in this work by their large size, almost filling the whole 2 inches square field at 3,600 times; by their characteristic oval shape; by the centric position and the diffuse, elliptical outline of the nucleus within them; by the proportion of their cytoplasmic to nuclear area; by the homogeneity of their cytoplasm and

<sup>1</sup> Wolpers, C., *Naturwiss.*, 1941, **29**, 416.

<sup>2</sup> Jung, F., *Klin. Wochsch.*, 1942, **21**, 917.

<sup>3</sup> Rebuck, J. W., and Woods, H. L., *Blood*, 1948, **3**, 175.

<sup>4</sup> Rebuck, J. W., Woods, H. L., and Monaghan, E. A., *Proc. Soc. Exp. Biol. and Med.*, 1948, **68**, 220.

<sup>5</sup> Barnes, R. B., Burton, C. J., and Scott, R. C., *J. Appl. Phys.*, 1945, **16**, 730.

<sup>6</sup> Jones, W. M., *J. Sci. Instr.*, 1947, **24**, 113.

<sup>7</sup> Heinmetz, F., *J. Bact.*, 1948, **55**, 823.

<sup>8</sup> Dounce, A. L., *J. Biol. Chem.*, 1943, **147**, 685.

<sup>9</sup> Hoerr, N. L., *Biol. Symposia*, 1943, **10**, 185.

(Fig. 1, 2 and 3) were seen to be extremely flat with outline reminiscent of that of a fried egg. From considerations of electron scattering from such shapes it is very unlikely that the dark outlines in Fig. 1, 2 and 3 can be interpreted as arising from a cell wall or a cell membrane. On the contrary, the most probable explanation for the dark outline and for its non-uniform thickness is that they arise from adsorbed or other material caught about the edge of the cell area as it dries. However, the fact that there is a discrete cell area at all in the micrographs indicates that a cell membrane may exist, although it is invisible in the micrographs. The dark outlines are not visible in the partially hemolyzed or the fixed erythrocytes although the cell areas are again quite discrete. This is evidence, coupled with the previous observations that neither the partially hemolyzed nor the fixed cells flatten out on the films to the same extent that the well-hemolyzed erythrocytes do, and it also suggests again that a cell membrane exists for an erythrocyte, but that it is thin enough physically and atomically to produce negligible electron scattering. Further evidence for the existence of a membrane is given later in the discussion on fixed erythrocytes.

The observations cited so far concerning hemolyzed and partially hemolyzed, unfixed erythrocytes tend to support the important concept of erythrocyte structure, that there is a cell membrane. They also support the following hypotheses concerning the mechanism of erythrocyte hemolysis: (1) that at partial hemolysis an amount of hemoglobin leaves the cell sufficient to allow some electron transmission, so that a spongy appearance becomes manifest over the cell area; at this time (Fig. 4 and 5), no cell membrane is seen, the cell is still distended and a nucleus is dimly visible, and (2) that at the more advanced stages of hemolysis the spongy content contracts upon the nucleus increasing its opacity but leaving a clear area around it, the cell flattens due to lack of cell content and adsorbed material collects about the edge of the cell area as it dries.

In spite of the obvious distortions which are introduced by the fixation (see below) erythrocytes in fixed preparations are recog-

nized and distinguished from the nucleus by use of the same criteria as they are in the unfixed samples. No significant, morphologic differences are detected between erythrocytes fixed with formalin but not stained and those fixed with formalin and stained with hematoxylin, methyl green or safranin. Fig. 10, 11 and 12 show erythrocytes which were fixed and stained with a variety of stains. Fig. 7, 8 and 9 are of a formalin-fixed, unstained sample. Erythrocytes in fixed specimens, both stained and unstained, are obviously distorted due chiefly to the fixation, since unfixed cells are not observed to shrink in the electron microscope at the low intensities at which these were examined. Marked shrinkage of the erythrocytes was also noted in the fixed specimens examined with the light microscope where there was no drying.

Except for the existence of a nucleus, fixed erythrocytes resemble empty bladders in electron micrographs (Fig. 7 to 12), and are similar in this respect to the non-nucleated erythrocytes studied by Wolpers<sup>1</sup> and Jung.<sup>2</sup> The fixed erythrocytes show foldings which are frequently radiated and which indicate that the erythrocytes possess an envelope or membrane. In mammalian erythrocytes at least, this envelope apparently corresponds to the plasma membrane with very little cytoplasm.<sup>10</sup> Such foldings would not be easily seen and indeed are not observed in wet preparations examined with the optical microscope, although they might exist in the wet. It seems logical to explain the occurrence of folds in electron micrographs by assuming that the fixed cell is flabby and its envelope loose. In the act of drying, the envelope falls together on the surface of the specimen film and the folds become visible. In the recently reported work of Chu, Dawson and Elford<sup>11</sup> electron micrographs of fixed chicken red cells are shown which exhibit morphology similar to that reported here.

The reticular structure of the stretched erythrocyte envelope depicted by Wolpers<sup>1</sup>

<sup>10</sup> de Robertis, E. D. P., Nowinski, W. W., and Saez, F. A., *General Cytology*, Philadelphia, W. B. Saunders Co., 1948, 122.

<sup>11</sup> Chu, C. M., Dawson, I. M., and Elford, W. J., *Lancet*, 1949, CCLVI, 602.

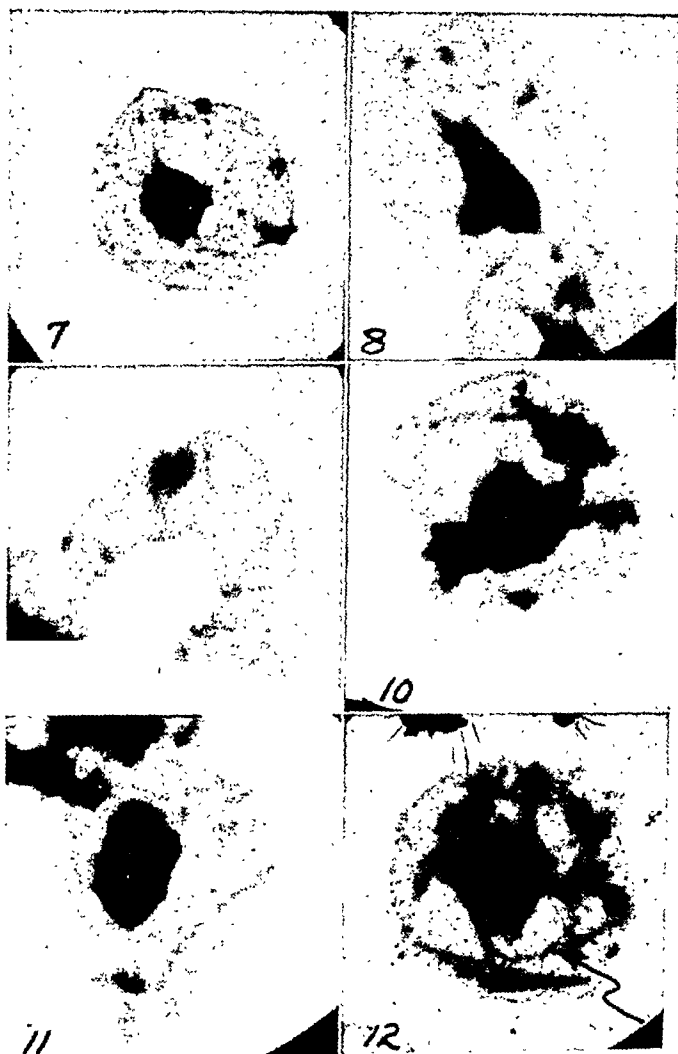


PLATE II.

Electron micrographs of osmosis-hemolyzed, chick embryo erythrocytes, formalin fixed.  $\times 3630$ .

Fig. 7, 8 and 9: Unstained

Fig. 10: Hematoxylin stained.

Fig. 11: Methyl green stained.

Fig. 12: Safranin stained.

and 5, but the cytoplasm of each of the hemolyzed erythrocytes in Fig. 1 to 5, inclusive, is somewhat transparent to electrons as compared with the known homogeneous opacity of unhemolyzed erythrocytes.<sup>3,4</sup> The sponge-like appearance of the erythrocytes in Fig. 4 and 5 may be the result of an incomplete hemolysis, but it could also support the theory that erythrocytes have a spongy struc-

ture. On the other hand, appearance of the images in Fig. 2, 3 and 7 to 12 would support the theory of a balloon structure for erythrocytes.

On several occasions it was possible to rupture the films which supported the erythrocytes and to observe their cross-sections as they were tilted upon them. Under these circumstances the well-hemolyzed erythrocytes

cytes clearly showed an envelope. Fixation and drying produced specimen changes, and in the specimens examined the processes of

electron microscopy provoked less radical changes than did the fixation.

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### 17285. Effect of Thymine Desoxyriboside (Thymidine) on Human Pernicious Anemia.

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Shive *et al.*<sup>1</sup> isolated from liver a crystalline substance that inhibited the antagonistic action of methyl-folic acid on the growth of *Leuconostoc mesenteroides* 8293. This substance was identified as thymidine, the desoxyriboside of thymine. Wright *et al.*<sup>2</sup> reported that thymidine could replace vitamin B-12 as a growth factor for certain lactic acid bacteria. Hypoxanthine, adenine or cytosine desoxyribosides<sup>3</sup> and guanine desoxyriboside<sup>4</sup> have also been shown to be able to replace vitamin B-12 as a growth factor for various bacteria.

From this evidence it seemed possible that vitamin B-12 may participate in the synthesis of desoxyribosides, essential to the formation of desoxyribose nucleic acids. Since thymine is effective in combating the hematologic lesions of pernicious anemia, nutritional macrocytic anemia and sprue,<sup>5</sup> the effect of thymidine on patients with pernicious anemia was investigated.

**Methods.** Three patients with Addisonian pernicious anemia in relapse were treated. All

of them had a macrocytic, high color index anemia, with gastric achlorhydria after histamine and megaloblastic hyperplasia of the bone marrow. Reticulocyte counts were done daily, and erythrocyte counts twice a week during the period of observation.

**Results.** In the first patient, a single injection of 5.3 mg of thymidine was followed by an increase of reticulocytes from 2.0 to 5.0% 4 days later, but by no significant rise in erythrocytes. One week after administration of the thymidine, daily intramuscular injections of 0.001 mg of vitamin B-12 were begun. There was a reticulocyte rise to 10.3% and the red blood cells rose from 2,430,000 to 4,250,000 in the next 13 days.

In the second patient a single injection of 150 mg of thymidine was followed by a rise in reticulocytes from 0.4% to 5.3% on the 4th day, but the red count failed to rise during the week following the injection. Daily intramuscular injections of 0.001 mg of vitamin B-12 were then started. Reticulocytes were 37% on the 7th day and the blood count had risen from 1,600,000 to 4,200,000 on the 21st day of this therapy.

The third patient received a sub-optimal intramuscular dose of 0.00025 mg of vitamin B-12 daily. Reticulocytes increased to 15.5% on the 8th day, and were 1.8% on the 17th day of therapy. At this time, in addition to the vitamin B-12, 5 mg of thymidine was given intramuscularly daily for 9 days. There was a secondary reticulocyte rise from 0.8% on the 18th day to 2.8% on the 21st, 22nd,

\* Died May 20, 1949.

<sup>1</sup> Shive, W., Eakin, R. E., Harding, W. M., Ravel, J. M., and Sutherland, J. E., *J. Am. Chem. Soc.*, 1948, **70**, 2299.

<sup>2</sup> Wright, L. D., Skeggs, H. R., and Huff, J. W., *J. Biol. Chem.*, 1948, **175**, 475.

<sup>3</sup> Kitay, E., McNutt, W. S., and Snell, E. E., *J. Biol. Chem.*, 1949, **177**, 993.

<sup>4</sup> Hoff-Jorgensen, E., *J. Biol. Chem.*, 1949, **178**, 525.

<sup>5</sup> Spies, T. D., and Stone, R. E., *Lancet*, 1947, **1**, 174.



offers a striking similarity to the cytoplasmic structure of some of the hystiocytes described by Rebuck and Woods.<sup>3</sup> Wolpers and Ruska<sup>12</sup> have reported a similar structure for the cytoplasm of the blood platelet hyalomere. One wonders about the relation of fixation and other technical processes to these appearances because in an electron microscope study of tissue culture cells, Porter, Claude and Fullam<sup>13</sup> observed a remarkable variation in the appearance of cytoplasm structure according to the fixation technic employed. Frey-Wyssling<sup>14</sup> points to the possibility that Wolpers' images show artifacts since a similar structure has been observed in the erythrocyte envelope denatured by heat hemolysis.<sup>2</sup>

The outlines of fixed erythrocytes are often observed to be angular. In Fig. 9 the outline is almost hexagonal and approximations to this particular shape were often encountered. The nuclei of fixed erythrocytes are also shrunken and distorted markedly, and are somewhat angular in outline. Nucleus distortions similar to those in the figures of Pl. II were observed in the control wet preparations. This suggests again that the causes of such distortion are to be found not as much in the act of drying as in the action of the fixative on cells which have previously suffered osmotic changes, since the nuclear distortions like the whole-cell distortions are not seen in fresh preparations when the sample is unfixed and osmotic changes have not occurred. The opacity of the nucleus to electrons is increased by the fixation, the contrast vis-a-vis nucleus and cytoplasm being improved thereby. However, this improvement in contrast offers no additional morphological information over unstained specimens; rather, detail is lost due to the increase in opacity of the nucleus. In most of the unfixed as well as the fixed erythrocytes, the nucleus is too opaque to electrons to evidence its structural details, but a coarse reticulum can be ob-

served in some of them in the unfixed samples, Fig. 3 and 5.

A reliable identification of erythrocytes could not be made in specimens which had been treated with either osmic acid or silver nitrate. Both fixatives produced almost homogeneously opaque, oval images in which a reticular structure was evidenced but only at the borders. These erythrocytes had also shrunk and the most marked shrinkage was observed in those treated with osmic acid.

Besides the observations of morphology which have been made in this work and reported in the foregoing discussion, there are a number of points concerning the electron microscopy of biological material<sup>15</sup> which have been illustrated: (a) a biased gun, if used properly and at less than maximum intensities does not affect the subject more than an unbiased one; (b) the primary heating effect of the electron beam is to shrink the cells, but this shrinkage from actual observation is not as drastic, nor does it introduce so many artifacts as does the process of fixation under the experimental conditions; (c) the processes of fixing and staining here tended to complicate the interpretation of electron microscope images rather than to simplify or clarify it; and (d) in general, electron images were likely freer of artifact than light microscopic images when unfixed, unstained samples were used in the former and fixed, stained samples in the latter.

*Summary.* Electron micrographs of osmosis-hemolyzed erythrocytes from healthy chick embryos are presented. The cells are examined unfixed, unstained; formalin-fixed, unstained; formalin-fixed, hematoxylin stained; formalin-fixed, safranin stained; formalin-fixed, methyl green stained; osmic acid fixed, unstained; and silver nitrate-fixed, unstained. Unfixed, unstained erythrocytes prepared by the technic employed showed a transparent cytoplasm so that the direct visualization of intracytoplasmic parasites might be possible by this procedure. Some nuclear structure was observed, and there is evidence for the existence of a cell membrane. Fixed erythro-

<sup>12</sup> Wolpers, C., and Ruska, H., *Klin. Wochsch.*, 1939, **18**, 1077, 1111.

<sup>13</sup> Porter, K. R., Claude, A., and Fullam, E. F., *J. Exp. Med.*, 1945, **81**, 233.

<sup>14</sup> Frey-Wyssling, A., *Submicroscopic Morphology of the Protoplasm and its Derivatives*, New York, Elsevier Publishing Co., Inc., 1948, 173.

<sup>15</sup> Watson, J. H. L., *J. Appl. Phys.*, 1948, **19**, 713.

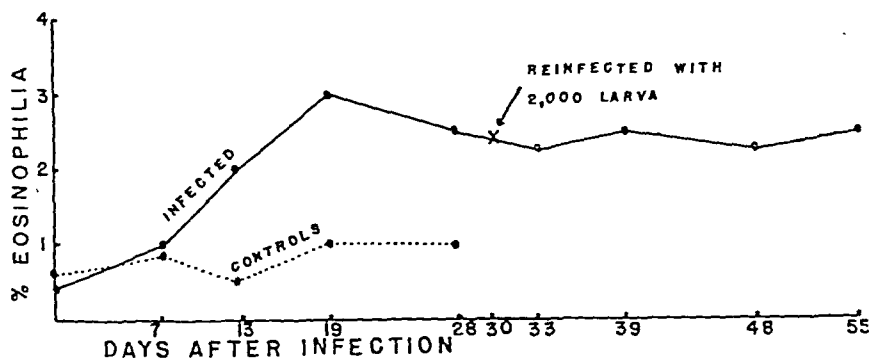


FIG. 1.

Eosinophil response of intact rats infected with *Trichinella spiralis* larvae, as compared with intact uninfected controls.

humans (Gould<sup>5</sup>) and has been reported rather high for mice (Hunter and Groupé,<sup>6</sup> Stein<sup>7</sup>) and rats (Somerén<sup>8</sup>). This suggested that the eosinophil response in intact and adrenalectomized animals infected with this parasite would yield interesting results. Thus the following study was undertaken.

**Experimental.** Preliminary experiments were performed to check the eosinophil response of our rats to *T. spiralis* infection. These animals (Wistar strain) were raised and maintained on our stock diet in an air-conditioned room (76°F). The experimental procedures for infecting the animals with standard doses of larvae have been described elsewhere (Larsh and Kent<sup>9</sup>). It is important to point out that the viability of these larvae in all experiments was checked by determining in a few control animals the percentage development of adult worms 5 days post-infection. The blood for eosinophil counts was obtained from the tail, stained with Giemsa stain in the usual manner, and examined under oil immersion. These counts were done at approximately weekly intervals.

Sixteen rats (2.5 months old) were used to determine the eosinophil response. Three re-

ceived 500 larvae; three, 1000 larvae; three, 1500 larvae; three, 2500 larvae; and 4 were kept as uninfected controls. The infected animals were reinfected with 2000 larvae 30 days after initial infection. Since the eosinophil response was similar for all of the infected animals, the results were plotted as the same (Fig. 1). The graph shows very little eosinophilia produced in these animals, as contrasted to the 20% (in 16 days) mentioned above (Somerén<sup>8</sup>). Our results, however, compare favorably with those of Beahm and Downs.<sup>10</sup> It is unlikely that our results were due to a strain factor in the rats in that random counts of another rat strain infected as above revealed about the same findings. It is possible that our strain of *T. spiralis* is a poor stimulator of eosinophilia. There is some support for this in that our white mice (2.5 months old), likewise, showed a poor eosinophil response. These were infected with various doses of *T. spiralis* larvae; 3 with 50 larvae; 3, 100 larvae; 3, 300 larvae; and 3, 400 larvae. All of these showed about the same level of eosinophilia, which reached a maximum of 10% after one month. The 4 non-infected controls averaged about 3%. The response of the infected mice was considerably lower than that (25-33% within 2 weeks) reported by the above mentioned workers.<sup>6,7</sup>

Despite the poor eosinophil response described above, it was decided to determine

<sup>5</sup> Gould, S. E., *Trichinosis*, 1945, C. C. Thomas Co., Springfield, Ill.

<sup>6</sup> Hunter, G. W., III, and Groupé, V., *J. Parasitology*, (suppl.), 1939, 25, 33.

<sup>7</sup> Stein, K. F., *Anat. Rec.* 1949, 103, 508; *Proc. Soc. Exp. Biol. and Med.*, 1949, 71, 225.

<sup>8</sup> Someren, V. D., *J. Helminthol.*, 1938, 10, 83.

<sup>9</sup> Larsh, J. E., Jr., and Kent, D. E., *J. Parasitology*, 1949, 35, 45.

<sup>10</sup> Beahm, E. H., and Downs, C. M., *J. Parasitology*, 1939, 25, 405.

and 24th days. Injections of 0.00025 mg of vitamin B-12 were continued from the 27th to the 40th day. During the first period of vitamin B-12 alone the red blood cell count rose from 1,850,000 to 2,220,000. During the second period with thymidine added the count increased further to 2,800,000. At the end of the third period of sub-optimal vitamin B-12 alone it had decreased to 2,600,000.

**Discussion.** The replacement ratio of thymidine for vitamin B-12 is about 300:1 for the growth of *L. lactis*.<sup>6</sup> We have seen a maximal reticulocyte response in pernicious anemia to as little as 4  $\mu$ g of B-12.<sup>7</sup> A dose of 5 mg of thymidine should therefore be more than adequate if the ratios observed in the bacterial growth system obtain for man. (That this is so for thymine with reference to folic acid was shown by Spies and his associates.<sup>8</sup>) From the results we obtained, it appears that thymidine alone is incapable of sustaining blood regeneration, although it does appear to cause a slight increase in reticulocytes. Similar results were reported by Geerts and Lens using doses of 10 mg of thymidine a day for 3

days.<sup>8</sup> From our observations it is apparent that the response to 150 mg was no greater.

It has been suggested that crude liver extracts may contain substances that give an enhanced hematopoietic response compared to B-12, and thymidine may be one of these accessory substances.<sup>9</sup> From our study it appears that thymidine is not completely inert hematopoietically, but if it has an enhancing effect on B-12 activity, this effect is very slight in the dosage used in this study. In view of the ability of other desoxyribosides to substitute for B-12 in bacterial growth, the effect of mixtures of desoxyribosides on pernicious anemia should be investigated.

**Summary.** 1. In 3 patients with pernicious anemia, thymidine in doses of 5 to 150 mg was found to cause slight reticulocytosis, but no effect on the red blood count.

We are deeply indebted to Dr. Esmond E. Snell, who generously supplied us with the thymidine used in these studies, and Mr. W. S. McNutt, who prepared it.

<sup>8</sup> Geerts, S. J., and Lens, J., *Nature*, to be published.

<sup>9</sup> Jacobson, M., and Bishop, R. C., *J. Clin. Invest.*, 1949, **28**, 791.

Received June 8, 1949. P.S.E.B.M., 1949, **71**.

## 17286. Effect of Adrenalectomy on Eosinophil Response of Rats Infected with *Trichinella spiralis*.

JOHN E. LARSH, JR. AND JOHN NICHOLS. (Introduced by A. T. Miller, Jr.)

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Recently there have appeared reports<sup>1-4</sup> that adrenal hormones depress the number of circulating eosinophils. Injection of adrenal cortical hormone will cause a lowering

<sup>1</sup> Thorn, G. W., Forsham, P. H., Prunty, F. T., and Hills, A. G., *J. Am. Med. Assn.*, 1948, **137**, 1005.

<sup>2</sup> Forsham, P. H., Thorn, G. W., Prunty, G., and Hills, A. G., *J. Clin. Endocrinol.*, 1948, **8**, 15.

<sup>3</sup> Hills, A. G., Forsham, P. H., and Finch, C. A., *Blood*, 1948, **3**, 755.

<sup>4</sup> Hellman, L., *Science*, 1949, **109**, 280.

of the number of circulating eosinophils, or injection of adrenotropic hormone from the pituitary will accomplish the same result if the adrenal gland is intact. In fact, this lowering of the eosinophils taken together with other changes in the body chemistry is proposed as a test for adrenal cortical insufficiency. Presumably the presence of the adrenal cortex has an inhibitory influence on the eosinophil producing bone marrow.

The eosinophil response to infection with *Trichinella spiralis* usually is striking in:

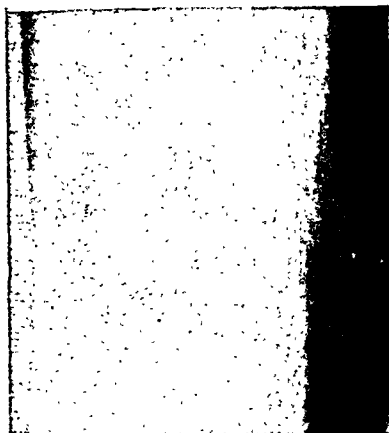


FIG. 1a.

The photograph demonstrates the vasoconstriction on the forearm of a subject 7 minutes after intradermal injection of 0.1 cc epinephrine 1:100,000.

FIG. 1b.

A sweat print of the same area as that in 1a 5 minutes after the injection. Note the lymphatic spread downward.

gland activity, and observed no blocking effect of dibenamine. Unfortunately, no tests were made with sympathomimetic drugs; furthermore, the results are not necessarily applicable to man. The present study followed the accidental observation of a local sweat response on the forearm of two subjects after the intradermal injection of epinephrine. After this study was underway the report of Kisin<sup>4</sup> appeared, stating that the subcutaneous injection of adrenaline, in concentrations as low as  $10^{-6}$ , produced local sweating.

**Methods.** In all experiments, Randall's iodine-starch paper method<sup>5</sup> has been used. Injections of approximately 0.1 cc were made with a 27 gauge needle into the skin of the volar surface of the forearm, except as noted otherwise. Observations were continued for 5 to 20 minutes after injection.

**Results.** Thirty subjects have been tested of whom nine showed no significant response to commercial, synthetic epinephrine hydro-

chloride (Winthrop Chemical Company), 1:10,000 or 1:100,000, in physiological saline; 5 of these were tested with acetylcholine or nicotine and gave a marked sweat response. On one occasion epinephrine 1:100,000 caused an apparent inhibition of sweating in an individual who had rather profuse spontaneous sweating. The remaining 21 subjects manifested definite sweating after epinephrine 1:10,000 to 1:1,000,000. The response varied widely in intensity among subjects, and in individual subjects on different days. In several experiments the solutions used were made up with crystalline epinephrine (Adrenalin, Parke, Davis & Co.) rather than from the stock solution, and results were identical. Members of both sexes, and of white, Negro and mongoloid races have been tested; no differences in responses of these groups were apparent.

The response, when present, commenced within a minute of the injection. The pattern of sweating closely approximated the area of vasoconstriction, and followed its local and lymphatic spread (Fig. 1). Secretion was observed up to 20 minutes, at which time the rate seemed to have diminished. Neosynephrine hydrochloride (Winthrop-Stearns) 1:10,000 or 1:100,000 produced sweating in

<sup>3</sup> Patton, H. D., *Proc. Soc. Exp. Biol. and Med.*, 1949, **70**, 412.

<sup>4</sup> Kisin, E. E., *Vestnik Venerol. i. Dermatol.*, 1948, No. 5, 27. (Abstracted in *Chem. Abstr.*, 1949, **48**, 2323).

<sup>5</sup> Randall, W. C., *J. Clin. Invest.*, 1946, **25**, 761.

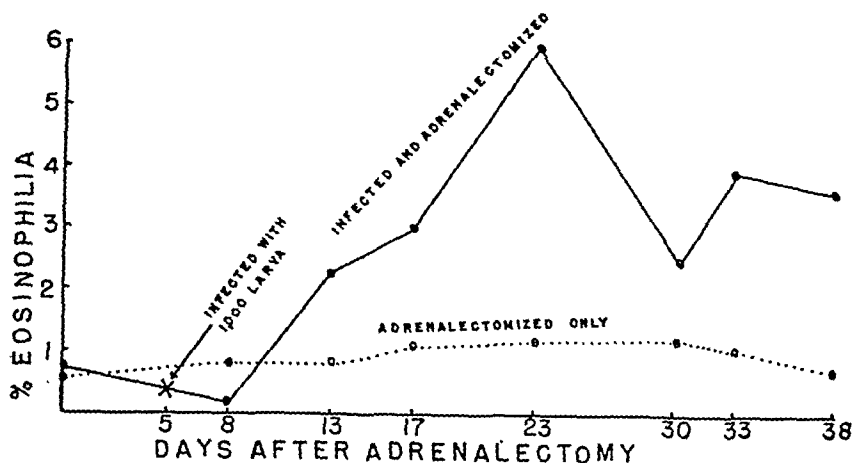


Fig. 2.

Eosinophil response of rats infected with *Trichinella spiralis* larvae 5 days after adrenalectomy, as compared with uninfected adrenalectomized controls.

the effect on this response of adrenalectomy. This work was limited to rats. Nineteen (2.5 months old) were adrenalectomized in a one stage operation, and kept on a potassium-low diet (Nichols<sup>11</sup>). They were given drinking water with 0.8% NaCl and 0.1% NaHCO<sub>3</sub>, and remained in apparent good condition throughout the experiment. The completeness of adrenalectomy was verified at autopsy. Eleven of these rats were infected with 1000 *T. spiralis* larvae 5 days after adrenalectomy, the remaining 8 were kept as uninfected controls. The eosinophil counts of all of the animals are shown in

Fig. 2. While the counts are somewhat higher than those shown in Fig. 1 for intact rats, the difference is too slight to draw definite conclusions. Perhaps it would be worthwhile to repeat this experiment with an animal which has a significantly higher granulocyte count than the rat.

**Summary.** (1) White mice and rats failed to show a high eosinophilia following initial infection with various doses of *T. spiralis*. (2) Rats also failed to elicit a striking response following reinfection. (3) Adrenalectomy influenced only slightly the number of circulating eosinophils in infected rats.

<sup>11</sup> Nichols, J., *Arch. Path.*, 1948, 45, 717.

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## 17287. Local Sweating in Man Induced by Intradermal Epinephrine.

RALPH R. SONNENSCHN. (Introduced by M. I. Grossman.)

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The possibility of an adrenergic innervation of the sweat glands was suggested recently by Haimovici,<sup>1</sup> who reported that intravenous injection of neosynephrine was followed by an increase in palmar sweating; this response was inhibited by dibenamine (N,N-dibenzyl-

$\beta$ -chloroethylamine hydrochloride). In preliminary experiments<sup>2</sup> in our laboratory, intradermal injection of neosynephrine into the palm failed to alter the pattern of spontaneous sweating. Patton<sup>3</sup> measured electrical potentials of the cat's paw, as an index of sweat

<sup>1</sup> Haimovici, H., *Proc. Soc. Exp. Biol. and Med.*, 1948, 68, 40.

<sup>2</sup> Janowitz, H., Sonnenschein, R. R., and Grossman, M. I., unpublished observations.

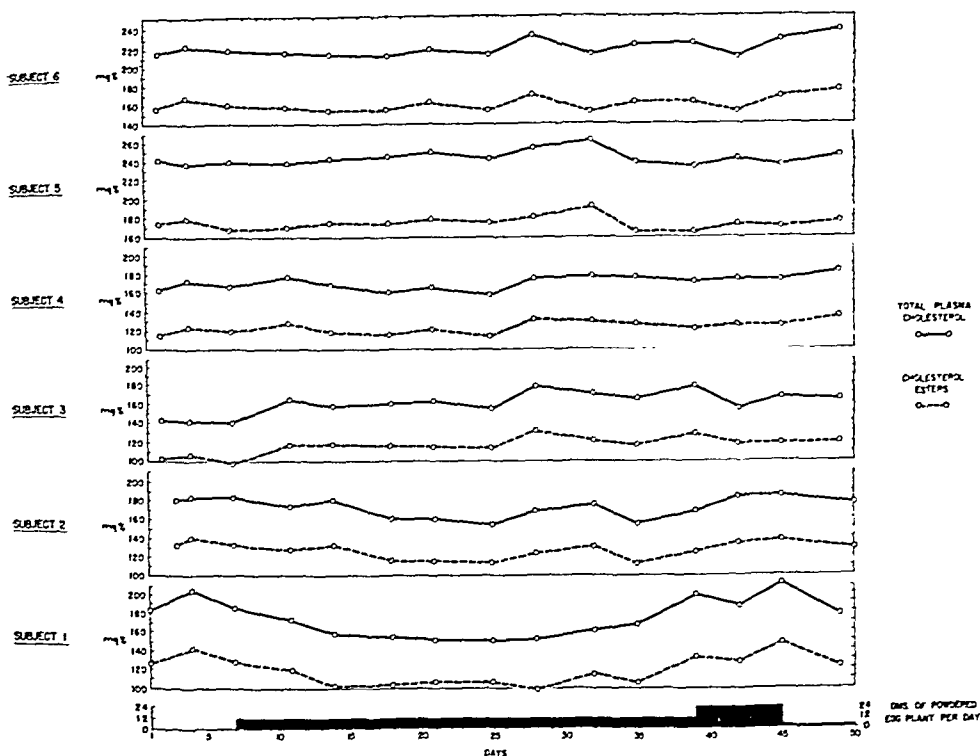


Fig. 1.

The values for both total plasma cholesterol in mg % and esterified plasma cholesterol in mg % are plotted individually for each of the six subjects against a common ordinate which shows the dosage of egg plant administered.

healthy males to determine its effect. One hundred fifty pounds of fresh egg plant was dried in a regular tunnel type drier; *i.e.*, the vegetable material was placed on trays and passed through the tunnel against a stream of air. Two stages of drying were used; *i.e.*, in the first stage a temperature of 145°F was maintained and the moisture content of the vegetable was reduced to approximately 12%. The vegetables were then removed from the trays in the tunnel and put into drying bins. Here the moisture was reduced from approximately 12% to approximately 4% and heat, not in excess of 130°F, was applied. This resulted in 9.5 pounds of slices which were powdered.

This powder was fed in doses of 12 g, and later 24 g a day, to six healthy males. Complete lipid fractionations on the blood plasma were done every third day for a control period of one week and throughout the

experiment. The values for free and esterified cholesterol are shown in Fig. 1. The remainder of the blood lipids likewise showed no change.

There were some unavoidable differences in the conditions under which our experiments were conducted and those of Roffo and Hainline. Roffo does not state how his material was dried nor does he give weights before and after drying. Hainline states that his was air dried but does not give weights before and after drying. Due to the pressure of other experiments upon our laboratory we were unable to use the egg plant for several months after it was delivered to us. It was however, hermetically sealed in light and water tight containers and kept in a cool place.

As can be seen, we observed no decholesterolizing effect upon any of the subjects. Subjects 1 through 4 had normal blood lipid values while subjects 5 and 6 are assumed to

4 individuals who responded to epinephrine, but the response was less intense. Three subjects who did not respond to epinephrine also failed to respond to neosynephrine.

Pretreatment of the skin by intradermal injection of 0.4 cc atropine sulfate 1:100,000 (4 subjects) or tetraethylammonium chloride 1:100 (2 subjects) had no significant effect on the response. At these concentrations, atropine has been shown to block the local action of acetylcholine, and TEA its axon reflex effects.<sup>6</sup> Procaine hydrochloride 1:100 (4 subjects), however, caused a slight to marked inhibition. Dibenamine was introduced by ion transfer\* into the skin of 2 subjects who had shown marked response to epinephrine. Twenty-four hours later, the treated areas of both subjects showed no response to epinephrine 1:1,000,000, while a definite effect was seen on the control arm.

<sup>6</sup> Janowitz, H., and Grossman, M. I., *Science*, 1949, **109**, 16.

\* 0.25 cc of a 5% solution of dibenamine was applied to an asbestos electrode of 7.5 sq cm. A current, whose density was 0.3 milliamp. per sq cm, was passed for 25 minutes. Performed through courtesy of Dr. Arthur A. Rodriguez, Department of Physical Medicine.

Epinephrine at 1:100,000 was only partially inhibited. The dibenamine had no significant effect on the response to acetylcholine chloride 1:1,000,000.

*Comment.* The epinephrine effect differs from that of acetylcholine in that the latter is characterized by (1) an associated axon reflex, (2) inhibition by atropine, (3) absence of inhibition by dibenamine.

These results indicate that at least some sweat glands of certain individuals are sensitive to the direct action of epinephrine but the physiological significance of this phenomenon is unknown. Experiments are in progress to elucidate the possible role of adrenergic fibers in regulation of sweat responses. Questions under consideration include the distribution of sweat glands which are sensitive to epinephrine, and their activity during thermoregulatory and emotional responses.

*Summary.* Intradermal injection of epinephrine caused local sweating in 21 of 30 subjects. The response was not altered by atropine or TEA but was diminished by procaine; dibenamine inhibited it. The physiological significance of this phenomenon remains to be elucidated.

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## 17288. Effect of Feeding Dried Egg Plant (*Solanum Melonga* L.) on Plasma Cholesterol.\*

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In a recent paper Roffo<sup>1</sup> stated that egg plant (*Solanum Melonga* L.) has a decholesterolizing effect in rabbits as well as in man. He also stated that it causes a diuresis. Hainline<sup>2</sup> has recently presented data which he

interprets as showing a like effect in rats.

Roffo has published graphs showing a decrease in serum cholesterol in rabbits. We were unable to find any data regarding his experiments with humans other than the statement that a like effect was noted. We have concluded that Hainline's interpretations are open to question, since a number of factors including trauma and infection were not controlled.

Because of our interest in finding some substance that would decrease the plasma cholesterol it was decided to feed dried egg plant to

\* The authors are grateful to the California Vegetable Concentrates, Inc., for furnishing as well as processing the egg plant.

<sup>1</sup> Roffo, A. H., *Yale Jr. Biol. and Med.*, Oct., 1945, **18**, 25.

<sup>2</sup> Hainline, A., Jr., Thesis presented to the faculty of the Graduate College, University of Denver, March 8, 1948.

After incubation, the nucleic acid and phosphoprotein were separated from all other phosphorus-containing substances, essentially according to the method of Schmidt and Thannhauser,<sup>7</sup> before counts were made. The contents of each flask were first repeatedly extracted with trichloroacetic acid until the supernatant solution did not count above background, and the supernatants were discarded. In a similar manner the residue was then extracted with a mixture of alcohol and ether and finally with a mixture of chloroform and methanol; the chloroform-methanol supernatant never did count above background.

The residue, containing only total nucleic acid and phosphoprotein phosphorus, was dried, weighed, and dissolved in alkali; an aliquot of this alkaline solution was then counted. Complete solution by alkali did not uniformly occur—in some samples a small part of the residue remained finely suspended. However, since duplicates which varied in degree of solubility showed no significant discrepancy in counts, the phenomenon was ignored.

*Results and discussion.* The results obtained are listed in Table I. Each result is the average of 2 determinations. Each sample was counted until a total of at least 2000 counts was obtained; thus the standard error of counting in each case is less than 3%.

The results show, in the case of liver and kidney tissue, that the incorporation of radiophosphorus into nucleic acid and phosphoprotein was inhibited in nitrogen, the magnitude of the inhibition averaging about 50-70%.

The fact that no significant difference between the percentage inhibition in normal tissue as a function of the presence or absence of glucose was observed indicates that under the conditions of the experiments the absence of added substrate was not a limiting factor. In other words, perhaps more than enough substrate for anerobic glycolysis was already present in liver and kidney.

On the other hand in tumor tissue a considerable decrease in the percentage inhibition was observed when the results with

TABLE I.  
Radiophosphate Uptake in Nucleic Acid and Phosphoprotein of Tissue Slices.

Tissue	Tumor				Kidney				Liver			
	% P <sub>32</sub> uptake in O <sub>2</sub>	% P <sub>32</sub> uptake in N <sub>2</sub>	% inhibition		% P <sub>32</sub> uptake in O <sub>2</sub>	% P <sub>32</sub> uptake in N <sub>2</sub>	% inhibition		% P <sub>32</sub> uptake in O <sub>2</sub>	% P <sub>32</sub> uptake in N <sub>2</sub>	% inhibition	
With glucose	4.7	3.6	24		2.1	0.76	75		2.6	0.83	69	
	4.0	3.4	15		2.4	0.93	62		3.2	2.1	40	
	8.4	8.3	1.5								61	
	4.2	2.8	12								32	
	1.6	1.2	22									
			Avg 15				Avg 69				Avg 51	
Without glucose	3.1	0.37	94		1.1	0.42	60		3.1	1.7	46	
	3.0	0.73	77		1.7	0.71	58				87	
	2.0	0.85	59								75	
	2.1	0.20	91						3.6	0.85		
			Avg 80				Avg 59				Avg 69	

<sup>7</sup> Schmidt, G., and Thannhauser, S. J., *J. Biol. Chem.*, 1945, **161**, 83.



have Essential Familial Hypercholesterol-

<sup>3</sup> Wilkinson, C. F., Hand, E. A., and Fliegelman, M. T., *Ann. Int. Med.*, Oct., 1948, **29**, 4.

emia<sup>3</sup> and to represent the heterozygous abnormal of this condition.

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# 17289. Synthesis of Nucleic Acid and Phosphoprotein in Normal and Cancer Tissue Slices Studied with Radio Phosphorus.\*

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Experiments have been performed using radioactive isotopes to study the synthesis of phospholipids and proteins in tissue slices and homogenates.<sup>1-6</sup> The synthesis of large molecules, *in vitro*, can be studied by the tracer technic even though they undergo a net degradation under the conditions of the tissue slice technic.<sup>1</sup>

In experiments reported here, radioactive phosphorus in the form of phosphate was used to study the synthesis of total nucleic acid and phosphoprotein in both normal and tumor tissue slices, by measuring the incorporation of radioactive phosphorus, under aerobic and anaerobic conditions and in the presence and absence of added glucose.

\* This paper is based on work performed under contract with the United States Atomic Energy Commission at the University of Rochester Atomic Energy Project, Rochester, N. Y., and is taken from University of Rochester Atomic Energy Report No. UR-20 (April, 1948).

<sup>†</sup> Now at Western Reserve University School of Medicine.

<sup>‡</sup> Now at University of Texas.

<sup>1</sup> Taurog, A., Chaikoff, I. L., and Perlman, I., *J. Biol. Chem.*, 1942, **145**, 281.

<sup>2</sup> Melchior, J., and Tarver, H., *Arch. Biochem.*, 1947, **12**, 309.

<sup>3</sup> Winnick, T., Friedberg, F., Greenberg, D. M., *Arch. Biochem.*, 1947, **15**, 160.

<sup>4</sup> Frantz, I. D., Jr., Loftfield, R. B., and Miller, W. W., *Science*, 1947, **106**, 544.

<sup>5</sup> Frantz, I. D., Jr., Zamecnik, P. C., Reese, J. W., and Stephenson, M. L., *J. Biol. Chem.*, 1948, **174**, 773.

<sup>6</sup> Friedkin, M., and Lehninger, A. L., *J. Biol. Chem.*, 1949, **177**, 775.

Briefly, the experiments indicate the dependence of the incorporation on the presence of oxygen, hence presumably on energy yielding reactions. The inhibition of phosphate incorporation in the absence of oxygen is partially prevented in tumor tissue, by the addition of glucose, thus suggesting that anerobic glycolysis can also provide the necessary energy.

*Experimental.* White rats were decapitated and the liver or kidneys removed. The slices cut were about 0.3 mm in thickness<sup>1</sup> and weighed about 25-30 mg when dry. They were placed in 50 cc Erlenmeyer flasks in 5 cc of Krebs-Ringer bicarbonate solution<sup>1</sup> containing radiophosphate. The solution had previously been equilibrated with the same gas mixture, either 95% O<sub>2</sub>:5% CO<sub>2</sub> or 95% N<sub>2</sub>:5% CO<sub>2</sub>, as was used in the flasks during the 2 hours of incubation with shaking at 37°C. The inhibition by N<sub>2</sub> was determined, in each case, on slices originating from the same organ or tumor.

The concentration of radioactive phosphorus in the Krebs-Ringer bicarbonate was approximately 1 microcurie per ml. In the experiments in which glucose was added to the Krebs-Ringer bicarbonate solution, its concentration was 0.2%.

Eleven normal rats and 9 rats bearing the transplantable carcinoma 256 (Walker tumor) were utilized. Care was exercised in sampling to avoid including any of the central necrotic regions of the tumor. The body weights of the normal rats employed were about 200 g, and of the tumor bearing rats, 120 g.

be used to cut sections as thin as  $0.1\ \mu$ , ours was especially made,\* hollow ground on both sides, with a long bevel.

**Fixing and Mounting Technics.** Several technics have been used to prepare tissues for microtomy. Fixation in about 4% neutral formalin, dehydration through graded alcohols, clearing in xylene, and imbedding in paraffin ( $85^{\circ}\text{C}$  melting point) has proved to be one of the most successful. With this method it is not necessary to "double imbed"; that is, to use both celloidin and paraffin.

The process of mounting a tissue section on a grid for electron microscopy varies according to the specimen and the operator, but in general the following technic has been successful. The section is transferred directly from the knife to a glass slide. A dissecting needle with a microscopic point is used to lift and move the sections. The point may be inserted slightly into the edge of the paraffin of the first section of a "ribbon" and the "ribbon" pulled out somewhat. A drop of warm water added to the section on the slide will further "spread" the section. When the water has dried, a drop of xylene may be added, or the whole slide may be immersed in xylene, to dissolve the paraffin from the tissue.

When the tissue is thoroughly dry, the slide is immersed in 2% collodion in amyl acetate which is thin dried in an even film. Lines are scored around the specimen; the slide is

breathed upon and immersed gently into water. The section adheres to the collodion film which strips from the glass slide and floats free. A grid is brought up beneath the specimen and both are lifted from the water. Thus the section and the supporting collodion film may be centered on the grid ready for electron observation.

Fig. 2 illustrates some results obtained with the equipment and technics described.

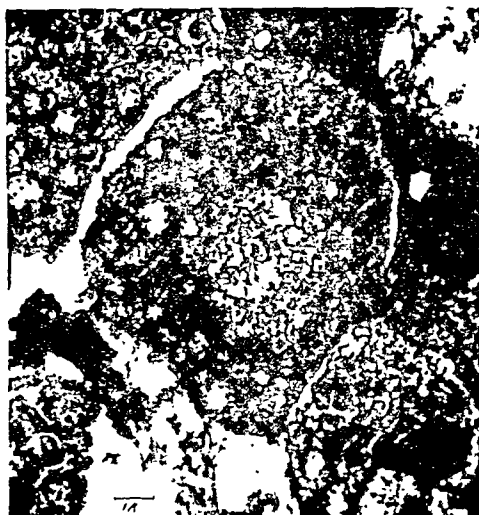


FIG. 2.

Section of rat intestine, sectioned at  $0.1\ \mu$ .

The greater part of the field is filled with a single cell. The nucleus appears less dense than structures seen in the cytoplasm. Individual structures have not been identified.  $\times 13,700$ .

\* Holzheimer, William, Melrose Park, Illinois.

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## 17291. The Rate and Total Loss of Body Water on the Survival Time of Adrenalectomized Frogs.\*†

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One aspect of the adrenal problem is the relation of the adrenal cortex to the regulation

\* This investigation was aided by the Comly-Coleman Fund of the Ohio State University.

† This work was originally initiated at our suggestion by Martin W. Williams.

of water and electrolytes in body fluids. The water content of certain tissues and, more pertinently, of certain cells (eviscerated carcass and liver of rats;<sup>1</sup> skeletal muscles of

<sup>1</sup> Silvette, H., and Britton, S. W., *Am. J. Physiol.*, 1933, 104, 399.

added glucose were compared with those without added glucose. This indicates, therefore, that at least in tumor tissue under anerobic conditions and in the presence of glucose, incorporation of the major portion of the radiophosphate may depend upon glycolysis. However, the evidence presented does not permit one to rule out the possibility that the remaining radiophosphate incorporation in liver and kidney slices under anerobic conditions is an "exchange" phenomenon independent of oxidative energy.

**Summary.** Phosphate incorporation into nucleic acid and phosphoprotein of liver, kidney and tumor tissue slices shows some dependence upon the presence of oxygen.

The inhibition of radiophosphate incorporation into nucleic acid and phosphoprotein of tumor slices by nitrogen and its partial reversal by the addition of glucose suggest that, at least in tumor tissue, glycolysis can also serve as a source of energy for the incorporation.

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## 17290. Low Speed Microtomy for the Electron Microscope.

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The beam of a 50-kv electron microscope does not penetrate specimens that are thicker than  $0.1\ \mu$ . Thus, in order to study biological specimens with this type of microscope we have further developed the technic described by Pease and Baker,<sup>1</sup> who altered a Spencer Rotary Microtome (model 820) by adding a wedge to the mechanism for forward movement. We reduced the angle of the inclined plane surface by a factor of 10 to 1 so that each step is  $0.1\ \mu$  rather than  $1.0\ \mu$ .

Since forward movement results from a pin in sliding contact with an inclined plane surface, it is important that the surface of the plane be as nearly perfectly flat as possible. The accompanying photograph, Fig. 1, illustrates the means by which the problem of a flat surface for even forward movement was solved. At the left of the picture (A) the original plane can be seen. The reduced angle is shown by D. The light gray triangular area (B) is an angle-reducing block of steel, to which is fastened an optical flat (C). A brass disc (E) instead of a pin is used for the feed screw tip. Horizontal movement of the disc is responsible for forward movement of the specimen. Vertical movement of the

flat and the mechanism to which it is attached causes the slicing action of the microtome. Since the disc and flat are held firmly in contact by a spring, any irregularities in the surface of the plane will be evident on the sliced section.

Since the ordinary microtome knife cannot



FIG. 1.  
Microtome.

- A. Original inclined plane surface of Spencer Microtome.
- B. Steel wedge reducing from  $25^\circ$  to  $2.5^\circ$  the angle of the original plane surface with respect to direction of the feed screw movement.
- C. Optical flat upon which cross feed disc rides.
- D. Altered inclined plane surface, making an angle of  $2.5^\circ$  with direction of cross feed movement.
- E. Cross feed disc.

<sup>1</sup> Pease, D., and Baker, R., *Proc. Soc. Exp. Biol. and Med.*, 1948, **67**, 470.

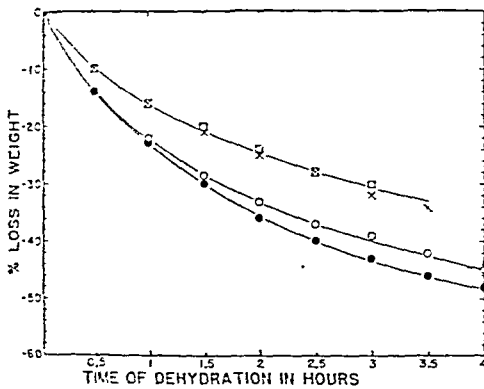


FIG. 1.

Percentage loss in body weights (water loss) of 4 groups of frogs plotted as functions of respective times (readings at  $\frac{1}{2}$  hour intervals) of exposure to a constant force of dehydration. The characters used to delineate the curves for each group are as follows: adrenalectomized frogs whose postoperative body weights were either uncontrolled (X), or controlled (□) to within  $\pm 1.5$  g of their respective preoperative values, and the control frogs—renal damaged (○) and unoperated (●). Volume of dehydrating system = 2230 ml, dry air current + 4.75 mm Hg. temp. 18–22°C.

absence of heart action and presence of fat bodies. In only 3 frogs of the 4 groups studied were feeble heart beats observed on autopsy which were not detected immediately before. These hearts failed to survive the half-hour interval following the close of the experimental run. These data are not included in the results presented.

**Results.** The various mean values for the percentage loss in body weights for the 4 groups of frogs are plotted in Fig. 1 as functions of time of exposure in hours to a constant dehydrating force. The means of the various data have been statistically compared for significance by "Student's" method and are presented together with their respective "t" values in Table I. The figure and table are self-explanatory after recourse to their accompanying legends.

When data from the 4 groups of frogs are analyzed for *a*, rate of loss and *b*, total loss of body weight, and *c*, duration of survival, the following orders of statistical significance are indicated (see Table I): the 2 groups of controls (renal damaged and unoperated) show no significance for the foregoing items

*b* and *c*, though *a* is probably significant. For reasons to be discussed, the renal damaged frogs are considered the controls for all subsequent comparisons. A comparison of data from the adrenalectomized frogs gives no significance for the aforementioned items *a*, *b*, and *c*. However, when either group of adrenalectomized frogs are compared with their controls, a high degree of significance is found for items *a* and *b*, and for item *c* as it affects the controlled but not the uncontrolled adrenalectomized frogs.

**Discussion.** The assumption is made here that any loss in body weight during the relatively short period of exposure to a constant force of dehydration is due to loss of body water.

The only significant difference between data from the 2 groups of controls (unoperated and renal damaged) lies in the respective rates of water loss. This difference is interpreted as due to the reduction in the total effective dehydrating surface of operated frogs arising from the encroachment of the thickened integument at the line of suture (4–5 cm) and of the loss in integument in effecting this suture. For this reason, the renal damaged frogs must be regarded as the true controls.

Since the difference between the respective means for data obtained from the 2 adrenalectomized groups is not significant, it remains to compare both of these groups with their controls. It has been shown that uncontrolled adrenalectomized frogs undergo postoperatively a progressive increase in body weight, so that, *e.g.*, on 7 and 12 days the mean body weight has increased by 28 and 33% respectively.<sup>9</sup> The 2 groups of adrenalectomized frogs when compared with the control show a highly significant decrease in the rate and in the total loss of body water; they differ between themselves in that the survival time is probably significant ( $P < 0.02$ ) for the controlled but not for the uncontrolled ( $P > 0.05$ ) adrenalectomized frogs. Were, possibly, the increase in osmotic pressure, resulting from forced dehydration, the underlying cause of this difference, then the uncontrolled, having the greater initial water load

<sup>9</sup> Angerer, C. A., unpublished data.

rats<sup>2</sup> and frogs,<sup>3</sup> and non-nucleated erythrocytes of dogs,<sup>4</sup> cats,<sup>5</sup> and rats<sup>6</sup>) is known to increase following adrenalectomy. Water shift in final analysis is the resultant of opposing osmotic forces acting across the plasma membrane of the particular cell in question. Since water shift, and more specifically osmotic pressure, is the variable under consideration, it appears from the very nature of the problem that an aquatic animal, like the frog, is the experimental material of choice. The frog imbibes water continuously through its integument when in an aqueous environment.<sup>7</sup>

The present studies were undertaken to determine the effect of known durations of exposure to a constant dehydrating force on *a*, rate and on *b*, total loss of body water, and on *c*, survival time of adrenalectomized frogs.

*Method.* Male frogs (*Rana pipiens*) weighing between 20 and 35 g and showing fat bodies on autopsy were used in these experiments. All frogs employed in this work may be conveniently divided into 4 groups (18-22 frogs/group): adrenalectomized frogs whose postoperative body weights were either (1) *uncontrolled*, that is, no attempt was made during the postoperative period to maintain the frog's weight at its preoperative value or (2) *controlled*, that is, the frog's postoperative body weight was maintained to within  $\pm 1.5$  g of its preoperative value, and the controls which consisted of both (3) *unoperated* and (4) *renal damaged* frogs.

Adrenalectomy was performed by "cold" cautery. Adrenal insufficiency was determined by the characteristic failure of the individual frog to perform successfully the righting reflex in not less than 3 and not more

than 5 successive attempts, after previous observation of its stance and color.<sup>8,9</sup>

Dehydration was effected by placing the desired frog in a closed system (Scheibler desiccator) of constant volume (2230 ml). This chamber was lined, except for the upper surface and a lateral window, with a dehydrating agent (anhydrous  $\text{CaCl}_2$ ). The system was continuously flushed during the experiment, except at the time of weighing of the frog, with washed, dried air entering via an 8-mm inlet under a pressure of 4.75 mm Hg. A given frog was placed in a closed wire basket of such size as to prevent excessive movements and of such design as to permit observation of all aspects of the body surface. This basket served both as a scale pan for it was suspended via a separate vent, provided for closure during the experiment, to a superimposed beam of an analytical balance, and as an electrode, for the basket was in circuit with 1 lead of an inductorium. The other electrode was stationary, though in such a position as to contact any portion of the ventral surface of the frog in its movable cage. A frog, after being placed in the desiccating chamber, was immediately weighed to the nearest 0.1 g. The moment of initial weight determination (elapsed time *ca.* 1 min.) was considered zero time. All subsequent weighings were made at half-hour intervals until the animal was declared dead (death point).

The death point was determined by the persistent absence of rhythmical heart beats. A very effective preliminary index, less arduous and thus less time-consuming, was the disappearance of skeletal muscle reflexes on faradic stimulation of the ventral integument with a pointed exploratory electrode, and also the less consistent, but more readily observed, buccal respiratory movements. The heart and buccal movements tend to accentuate as body volume decreases with continued dehydration. This favorable circumstance tends to offset the enfeebling of these movements prior to the terminal stage.

Autopsies were performed on all frogs immediately upon termination of an experimental run in order to confirm the continued

<sup>2</sup> Crismon, J. M., and Field, J., 2nd., *Am. J. Physiol.*, 1940, **130**, 231.

<sup>3</sup> Angerer, C. A., and Angerer, H. H., *Fed. Proc.*, 1942, **1**, 3.

<sup>4</sup> Harrop, G. A., *Bull. Johns Hopkins Hosp.*, 1936, **59**, 11.

<sup>5</sup> Hegnauer, A. H., and Robinson, E. J., *J. Biol. Chem.*, 1936, **116**, 769.

<sup>6</sup> Gonzalez Q., J., and Angerer, C. A., *Am. J. Physiol.*, 1947, **149**, 502.

<sup>7</sup> Adolph, E. F., *Physiological Regulations*, p. 110, The Jaques Cattell Press, Lancaster, Pa., 1943.

<sup>8</sup> Maes, J., *Arch. Intern. de Physiol.*, 1937, **45**, 135.

duction of free-moving particles or by a possible increase of osmotically-active particles, is to be considered as another example of stress with which the adrenalectomized organism fails to cope.

*Summary.* All frogs employed in this study may be conveniently divided into 4 groups (18-22 animals/group): the adrenalectomized frogs whose postoperative body weights were either controlled, to within  $\pm 1.5$  g of their individual pre-operative values, or uncontrolled; and the controls, both renal damaged and unoperated frogs. The individuals of each group were subjected to a constant dehydrating force and the resulting data were statistically analyzed with respect to the following points: *a*, the rate of loss and *b*, the total loss of body weight (water) and *c*, the duration of survival on exposure to a constant force of dehydration.

1. A comparison of the difference between respective means of the 2 groups of ad-

renalectomized frogs shows no significance as regards the foregoing items *a*, *b*, and *c*. 2. Comparison between the 2 groups of controls (renal damaged and unoperated frogs) gives no significance with respect to items *b* and *c*, though it does for *a*. 3. A comparison between the respective means for either group of adrenalectomized frogs and their controls (renal damaged) produces a significance for *a* and *b*, and for *c* as it affects the death point of the controlled but not of the uncontrolled adrenalectomized frogs. 4. On the basis of the known cardiovascular embarrassment subsequent to adrenalectomy, it is suggested that the increased osmotic pressure resulting from the forced water loss and the attendant decrease in peripheral circulation brings an increased osmotic stress to bear on an already weakened heart action. It is suggested that this stress is the deleterious factor in affecting the physiological points raised.

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## 17292. Use of Antitryptic Agents in Tissue Culture. I. Crude Soybean Trypsin-Inhibitor.\*†

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From the Connaught Medical Research Laboratories, University of Toronto.

Substances that inhibit the proteolytic activity of trypsin have been found in serum and plasma,<sup>1</sup> in egg white,<sup>2</sup> in navy beans and soybeans,<sup>3</sup> and in extracts of pancreas.<sup>4,5</sup>

\* This investigation was supported, in part, by grants from the National Cancer Institute of Canada, and the Ontario Cancer Treatment and Research Foundation.

† Grateful acknowledgement is made to Miss M. Ogilvie, Mrs. C. J. Porter, and Mr. C. J. MacFayden for technical assistance.

<sup>1</sup> Grob, D., *J. Gen. Physiol.*, 1943, **26**, 405.

<sup>2</sup> Balls, A. K., and Swenson, T. L., *J. Biol. Chem.*, 1934, **106**, 409.

<sup>3</sup> Bowman, D. E., *Proc. Soc. Exp. Biol. and Med.*, 1944, **57**, 139.

<sup>4</sup> Kunitz, M., and Northrop, J. H., *J. Gen. Physiol.*, 1936, **19**, 991.

<sup>5</sup> Kazal, L. A., Spicer, D. S., and Bralinsky, R. A., *J. Am. Chem. Soc.*, 1948, **70**, 3034.

Because of the great activity of these antitryptic agents, it seemed of interest to investigate the possibility of using them in tissue culture as a means of preventing the digestion of the plasma coagulum that frequently occurs during the growth of cells *in vitro*.<sup>6-8</sup> The soybean antitrypsin,<sup>†</sup> which has been

<sup>6</sup> Lambert, R. A., and Hanes, F. M., *J. Exp. Med.*, 1911, **13**, 495.

<sup>7</sup> Losee, J. R., and Ebeling, A. H., *J. Exp. Med.*, 1914, **19**, 593.

<sup>8</sup> Santesson, L., *Acta path. et microbiol. Scand.*, 1935, Suppl. **24**.

‡ It is interesting to note that Fischer has just reported (Fischer, A., *Science*, 1949, **109**, 611) a series of experiments with crystalline soybean trypsin inhibitor supplied by Kunitz.<sup>9</sup> His results are in complete accord with those reported here.

<sup>9</sup> Kunitz, M., *J. Gen. Physiol.*, 1947, **30**, 291.

TABLE I.

Summary and Comparison (*t*-test) of Mean Values for All Data (Line 3) Obtained from the Various Groups of Frogs Subjected to a Constant Force of Dehydration.

Frogs	Adrenalectomized						Control					
	1			2			3			4		
	Wt uncontrolled			Wt controlled			Normal			Renal damaged		
Experimental groups												
Statistical classes	a	b	c	a	b	c	a	b	c	a	b	c
	%	%	hr	%	%	hr	%	%	hr	%	%	hr
Mean	24.2	32.2	3.3	24.6	28.4	2.7	35.8	48.0	4.0	32.9	43.4	3.8
S.E. $\pm$	0.7	1.9	0.3	1.2	1.4	0.4	1.0	1.0	0.1	0.7	1.2	0.1
S.D. $\pm$	2.3	6.6	1.0	4.0	4.7	1.4	3.4	3.5	0.4	2.3	4.0	0.5
<i>t</i> -test												
	2	0.30	1.63	1.19								
		NS	NS	NS								
	4	9.32	5.07	1.55	6.20	8.43	2.54	2.46	1.53	1.09		
		HS	HS	NS	HS	HS	S	S	NS	NS		

Any given letter among the following (line 3) indicates the same variable studied in any group of frogs (lines 1 and 2) and this meaning is used throughout the text:

a = % loss of body weight at the end of 2-hour period of dehydration;

b = % total loss of body weight at death-point;

c = Duration of dehydration in hours until death-point.

S.E. = Standard error, and S.D. = Standard deviation of respective mean.

*t*-test = Statistical comparison of ratio of difference between an indicated pair of means/estimated standard error of this difference.

Statistically: NS = Not significant ( $P > 0.05$ ); S = Significant ( $P 0.05-0.01$ ); HS = Highly significant ( $P < 0.01$ ).

in comparison with the controlled adrenalectomized frogs, not only should survive longer but also should suffer the greater rate and the greater total loss of body water. None of these postulates is met on comparing the 2 groups of adrenalectomized frogs; but on comparing the latter groups with their controls the reverse tends to be true. The conclusion reached is that the adrenalectomized frogs can tolerate relatively slight loss in body water before lethal effects are encountered. This is not due to any decrease in permeability of the integument of adrenalectomized frogs, for all evidence points to an increase in permeability<sup>10</sup> in general and for frog skin<sup>9</sup> in particular.

The decreased rate of dehydration found in both groups of adrenalectomized frogs may be interpreted as due to the immediate decrease in replenishment of body fluid at the body surface. This condition may arise from the hemoconcentration and hemostasis known to occur in peripheral vessels as a result of adrenocortical insufficiency or ablation.<sup>11</sup>

Physiologically, forced dehydration has much in common with sweating but without benefit of the concomitant vasodilatation arising from the increase in environmental temperature. Sweating, and dehydration, like adrenocortical insufficiency, leads to a further decrease in plasma volume.<sup>12</sup> Thus, a decreased plasma volume, together with an increased blood viscosity and osmotic pressure, has a deleterious effect on an already weakened heart action<sup>13</sup> which follows in the wake of adrenalectomy.

That mammals deficient in adrenal cortical hormone are less able to withstand various forms of stress (certain types of drugs, poisons, toxins, infections, variations in environmental temperature, barometric pressure, and traumatic procedures) is too well-known to require elaboration.<sup>14</sup> Thus, an increase in osmotic pressure induced either by a re-

<sup>12</sup> Adolph, E. F., *Physiology of Man in the Desert*, p. 170, Interscience Publishers, Inc., New York, N. Y., 1947.

<sup>13</sup> Nicholson, W. M., and Soffer, L. J., *Bull. Johns Hopkins Hosp.*, 1935, **56**, 236.

<sup>14</sup> Swingle, W. W., and Remington, J. W., *Physiol. Rev.*, 1944, **24**, 89.

<sup>10</sup> Hartman, F. A., *Endocrinology*, 1942, **30**, 861.

<sup>11</sup> Swingle, W. W., Vars, H. M., and Parkins, W. M., *Am. J. Physiol.*, 1934, **109**, 488.

their controls were incubated for 7 days and were observed each day for signs of digestion of the coagulum. It was found that antitrypsin levels between 5.0 mg per ml and 0.25 mg per ml completely prevented digestion of the plasma coagulum, while lower concentrations were not effective. Fig. 1 and 2 show two representative cultures selected from this series and photographed at 11 days.

Comparable results were also obtained in other experiments in which fibrinogen-thrombin clots were used. In these instances, the effective concentrations of antitrypsin were found to be between 5.0 and 0.25 mg per ml.

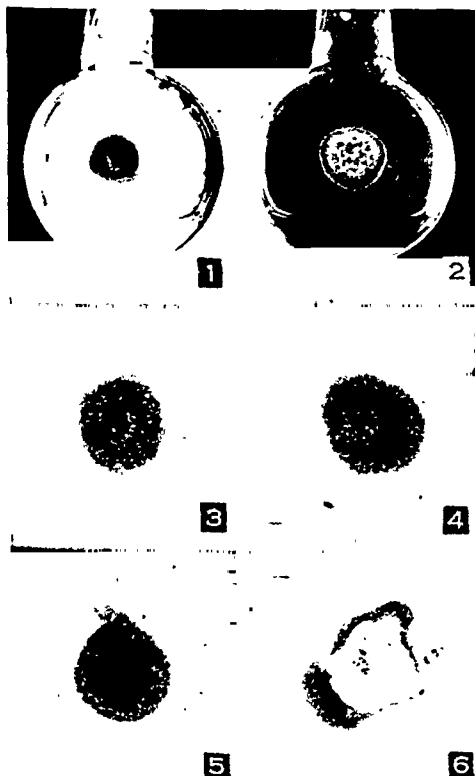


Fig. 1 and 2.

Eleven-day sister cultures of 12th passage chick fibroblasts cultivated in plasma medium in the presence of 0.5 mg antitrypsin per ml (Fig. 1), and in the absence of antitrypsin (Fig. 2).  $\times 1$ .

Fig. 3, 4, 5 and 6.

Seven-day sister cultures of 15th passage chick fibroblasts cultivated in plasma medium in the presence of 1.0 mg antitrypsin per ml (Fig. 3), 0.5 mg per ml (Fig. 4), 0.25 mg per ml (Fig. 5), and in the absence of antitrypsin (Fig. 6).  $\times 3$ .

In both types of coagulum, control cultures usually gave evidence of initial digestion within 24 to 48 hours. This appeared first as a thin ring in the medium a short distance from the tissue fragment, and progressed rapidly to form a crater containing liquefied plasma. The tissue fragment contracted very considerably, and in most cases was found floating freely in the liquid plasma. Cell growth frequently occurred on the surface of the glass beneath the digested area.

*Inhibition of plasma coagulation by antitrypsin.* It was found that the presence of high concentrations of the crude trypsin-inhibitor (5.0 to 1.0 mg per ml) prevented normal plasma coagulation in the presence of embryo extract. The addition of a few drops of dilute thrombin overcame this inhibition and permitted normal clot formation. This finding is in agreement with the results of Tagnon and Soulier,<sup>13</sup> who reported that both crude and crystalline antitrypsin from soybeans had a marked anticoagulant effect upon whole blood and recalcified plasma, but had no antithrombic activity. In the present investigation, it was also noted that the anti-clotting activity of trypsin-inhibitor was more pronounced when heparin or citrate was present in the plasma.

*Inhibition of tissue growth by antitrypsin.* The presence of crude soybean antitrypsin was found to retard the growth of the culture strains that were used as test material. The effect was most pronounced at high concentrations, 3.0 to 1.0 mg per ml. Cultures maintained in such high antitrypsin concentrations were observed to have a total area that was 20 to 30% less than the area of sister cultures grown in the absence of antitrypsin. The toxicity was less evident at lower levels, 0.5 to 0.25 mg per ml. Inhibition of growth was not uniform, however, in all cultures tested; and in some experiments the inhibition was negligible even at concentrations of 3.0 mg per ml. The effect of graded levels of crude antitrypsin on 15th passage strain cultures is shown in Fig. 3, 4, 5 and 6.

Repeated passage of cultures through media

<sup>13</sup> Tagnon, H. J., and Soulier, J. P., *Proc. Soc. Exp. Biol. and Med.*, 1946, **61**, 440.



shown by Kunitz<sup>9</sup> to be a protein of globulin nature, was selected as the first agent to be studied in the present investigation.

**Methods.** Crude trypsin-inhibitor was prepared from solvent-extracted unheated soybean flakes<sup>§</sup> by the method of Kunitz.<sup>10</sup> This material was dried from the frozen state, and the resulting powder was kept in the refrigerator. The activity of each preparation was determined by its ability to prevent the digestion of casein by trypsin, according to the procedure of Kunitz.<sup>9</sup> In these tests, graded amounts of antitrypsin were added to a standard solution of trypsin and the degree of proteolysis was then measured colorimetrically as tyrosine liberated by Folin's reagent. For use in tissue cultures, the dried powder was dissolved in 0.01 N HCl, and the material was sterilized by passage through a UF fritted glass filter. Stock solutions were diluted further in sterile glass-distilled water to the desired concentrations. All sterile solutions were kept in the refrigerator, and were found to retain their antitryptic potency for a period of at least 6 to 8 months.

Culture strains of fibroblast-like cells were derived from the leg muscle of 11-day chick embryos, and were cultivated in D-3.5 flasks through at least 10 to 12 passages (weeks) before use. In some experiments, use was made of fresh tissue explants.

The coagulum added to each flask consisted of 0.3 ml chicken plasma, 0.3 ml chick embryo extract, 0.3 ml Earle's modification of Tyrode's solution,<sup>11</sup> and 0.1 ml phenol red (0.025% in Earle's solution). In addition, a feeding mixture was added to each culture flask on the second day and was renewed on the fourth and sixth days. This feeding mixture was comprised of 0.3 ml embryo extract, 0.6 ml Earle's solution, and 0.1 ml phenol red solution. At no time was the original coagulum reinforced by the addition of another layer of plasma.

§ Obtained through the courtesy of Dr. W. D. McFarlane, Canadian Breweries, Ltd., Toronto, Ontario.

<sup>10</sup> Kunitz, M., *J. Gen. Physiol.*, 1946, **29**, 149.

<sup>11</sup> Earle, W. R., *J. Nat. Cancer Inst.*, 1943, **4**, 165.

Embryo extract was prepared by grinding 11-day chick embryos that were suspended in a roughly equivalent volume of Earle's solution in a graduated all-glass homogenizer. After the cell debris had been separated by centrifuging, the supernatant was frozen and thawed twice. This concentrated extract was diluted in Earle's solution to 1 part in 4 and was kept as a stock solution in the refrigerator. The stock solution was further diluted in Earle's solution to a final concentration of 1 part in 20 immediately before use.

The antitryptic factor (0.1 ml of an appropriate dilution) was added to both the original coagulum and the feeding mixture in place of 0.1 ml of Earle's solution. An equal volume of glass-distilled water was added to all control cultures.

In some experiments, the effect of antitrypsin was tested on cell colonies cultivated in fibrinogen-thrombin clots prepared by the method of Porter and Hawn.<sup>12</sup> To these cultures, 0.3 ml of serum was added to provide the nutrient materials ordinarily supplied by the plasma.

When the tissue transplants were prepared from strain material, each of the cultures chosen for an experiment was cut into 4 fragments of approximately equal size, and 3 of the fragments were embedded in separate flasks containing graded amounts of antitrypsin. The fourth fragment in each set served as a control and was cultivated in the absence of antitrypsin. Each experiment consisted of at least 3 sets of cultures containing the same ingredients.

To test for possible inhibition of migration and growth by the antitryptic agent, outline drawings showing the increase in surface area were made daily by means of a projectoscope, and the increments were measured with a planimeter.

**Results. Prevention of clot digestion.** In preliminary experiments, crude antitrypsin, prepared as described, was added to a series of 12th passage sister cultures at final concentrations of 5.0, 3.0, 2.0, 1.0, 0.5, 0.25, 0.1, and 0.05 mg per ml. These cultures together with

<sup>12</sup> Porter, K. R., and Hawn, C. v. Z., *Proc. Soc. Exp. Biol. and Med.*, 1947, **65**, 309.

# 17293. Studies of Experimental Pulmonary Edema. I. Pulmonary Edema from *l*-Epinephrine and *l*-nor-Epinephrine (Arterenol).<sup>\*</sup>

EATON M. MACKEY AND ELDEN F. PECKA, JR.

From the Scripps Metabolic Clinic, La Jolla, Calif.

Large doses of epinephrine result in death, accompanied by an acute pulmonary edema in various species.<sup>1-3</sup> Studies of pulmonary edema caused by a number of compounds suggested that the mechanism may be by way of the release of pressor amines from the adrenal medulla. The recent discovery that this organ contains<sup>4-9</sup> and releases *l*-nor-epinephrine<sup>10</sup> as well as *l*-epinephrine made it desirable to compare the production of pulmonary edema by these compounds.

**Experimental.** Unfasted guinea pigs were used with equal sex distribution in the various groups. The *l*-epinephrine<sup>†</sup> and the *l*-nor-epinephrine (*l*-arterenol) were administered as the hydrochlorides in 0.9% NaCl in concentrations near 0.1%. In those experiments in which it was used, the adrenergic blocking agent, N-(9-fluorenyl)-N-ethyl- $\beta$ -chloroethylamine-HCl ("SKF-501")<sup>‡</sup> was dissolved

in saline as a 0.1% solution for doses of 2 mg/kg 10-15 minutes before the pressor amine. The solutions were given intravenously via the penile vein in males and intracardially in female animals. There were 6 guinea pigs in each group. The degree of pulmonary edema was determined by the weight of the lungs.<sup>3</sup>

**Results.** Our data are summarized in Table I. The *l*-epinephrine, except in the dose of .001 mM/kg (from which they recovered and were sacrificed with ether an hour later), was rapidly fatal. Groups 3 and 4 survived an average of 11 and 4 minutes respectively. On the basis of previous experience, *l*-nor-epinephrine was used in larger doses than the epinephrine but although the higher doses (Groups 8 and 9) prostrated the animals, they recovered and were etherized an hour after the drug was given. Higher doses of *nor*-epinephrine were required to cause pulmonary edema and with the highest dosage used, .004 mM/kg, it failed to exceed that which resulted from one-fourth as much epinephrine.

These results are in accord with those of Tainter, Tullar and Luduena<sup>11</sup> who found that the LD<sub>50</sub> of intravenously administered *l*-nor-epinephrine in mice is eight times less than that for *l*-epinephrine. Undoubtedly these results were in part due to pulmonary edema.

Stone and Loew,<sup>3</sup> and others have shown that certain adrenergic blocking agents are capable of reducing epinephrine-induced pulmonary edema in animals and this effect has been used as one measure of the effectiveness of adrenergic blocking agents. We administered N-(9-fluorenyl)-N-ethyl- $\beta$ -chloroethylamine ("SKF-501"), a  $\beta$ -haloalkylamine, 10-15 minutes before the pressor amines. It prevented the occurrence of any symptoms whatever or of grossly measurable pulmonary edema otherwise induced by toxic doses of either *l*-epinephrine or *l*-nor-epinephrine.

\* Supported by a grant from the Life Insurance Medical Research Fund.

<sup>1</sup> Meltzer, S. J., *Am. Med.*, 1904, **8**, 191.

<sup>2</sup> Emerson, H., *Arch. Int. Med.*, 1909, **3**, 368.

<sup>3</sup> Stone, C. A., and Loew, E. R., *Proc. Soc. Exp. Biol. and Med.*, 1949, **71**, 122.

<sup>4</sup> Schümann, H., *Klin. Wschr.*, 1948, **20**, 37.

<sup>5</sup> Holton, P., *Nature*, 1949, **163**, 217.

<sup>6</sup> Pitcairn, D. M., and Youmans, W. B., *Fed. Proc.*, 1949, **8**, 127.

<sup>7</sup> Euler, U. S. von. and Hamberg, U., *Nature*, 1949, **163**, 642.

<sup>8</sup> Goldenberg, M., Faber, M., Alston, E. J., and Chargaff, E. C., *Science*, 1949, **109**, 534.

<sup>9</sup> Tullar, B. F., *Science*, 1949, **109**, 536.

<sup>10</sup> Bulbring, E., and Burn, J. H., *Nature*, 1949, **163**, 363.

<sup>†</sup> The *l*-epinephrine (Adrenalin) used was a specially purified sample of the natural product for which we are indebted to Dr. Leon A. Sweet of Parke, Davis & Company. It contained only a trace of *l*-nor-epinephrine (arterenol).

<sup>‡</sup> We are indebted to Dr. Glen E. Ulliot of the Smith, Kline & French Laboratories for generous supplies of "SKF-501."

<sup>11</sup> Tainter, M. L., Tullar, B. F., and Luduena, F. P., *Science*, 1948, **107**, 39.

containing inhibitory levels of antitrypsin resulted in a gradual disappearance of the growth-depressing effect. The cultures acquired a tolerance to antitrypsin after 3 to 5 passages, and thereafter appeared to grow normally in the presence of concentrations that had previously been found to be inhibitory.

*Effect of trypsin-inhibitor on fresh tissue explants.* When a study was made of the effect of several concentrations of crude soybean antitrypsin on fresh tissue explants in their first culture passage, it was found that levels of 1.0, 0.5, and 0.25 mg per ml completely prevented digestion of the plasma coagulum. It was also noted that fresh tissues were much less sensitive to the toxic action of antitrypsin than were strain cultures, and concentrations as high as 3.0 mg per ml had no appreciable inhibitory effect. This absence of growth inhibition was also observed in cultures of fresh tissues carried in fibrinogen-thrombin clots.

*Discussion.* Digestion of the plasma coagulum by growing cells has presented a considerable problem since the earliest days of tissue culture. Thus, Losee and Ebeling,<sup>7</sup> working with human culture material, attempted to prevent liquefaction of the plasma by the addition of serum, agar and egg albumin. Although these experiments were unsuccessful, it was found that dilution of the plasma with an equal volume of Ringer's solution slowed down the rate of digestion sufficiently to allow short term experiments to be carried out. Carrel<sup>14</sup> reported that he was able to protect fibrin clots from digestion by the addition of a little serum, a small amount of sodium linoleate, or a suspension of egg yolk. Of the various sera that have been employed, horse serum has proved to be most suitable, although it has the undesirable property of retarding the rate of cell multiplication. In many laboratories, the proteolytic action of tissues cultivated in plasma is controlled by the addition of a second layer of plasma, on the day following preparation of the cultures. Subsequent layers of plasma are then added as they become necessary. This procedure

takes considerable time and requires large amounts of plasma.

Some investigators<sup>6,14</sup> have attributed digestion of the plasma coagulum to proteolytic enzymes secreted by the cells during active growth. Others<sup>15-17</sup> have advanced the hypothesis that cells in tissue culture elaborate an activating substance that transforms an inactive precursor, profibrinolysin, present in the medium, into an active proteolytic enzyme, fibrinolysin, which then digests the clot. The present observations can be interpreted on the basis of either of these hypotheses.

The results reported in the present paper indicate that the incorporation of antitryptic agents in the culture medium offers promise of a solution to the troublesome problem of clot digestion. It is realized, of course, that the slight inhibition of growth caused by crude soybean antitrypsin presents a serious obstacle to its routine use. But further studies are now in progress with more highly purified preparations of the trypsin-inhibitor in an effort to eliminate the growth-retarding factor. It is also planned to extend the present investigation to a study of antitryptic agents derived from other sources.

*Summary.* Crude soybean trypsin-inhibitor has been found to prevent digestion of plasma and fibrinogen-thrombin clots by tissues growing *in vitro*. The presence of antitrypsin retards the normal coagulation of plasma but this inhibition can be overcome by the addition of thrombin. The growth of strain cultures of fibroblast-like cells is somewhat depressed by high concentrations of antitrypsin, but the inhibitory effect can be eliminated by repeated cultivation in antitrypsin. Fresh tissue explants are not as sensitive as strain cultures to this inhibitory action.

<sup>15</sup> Demuth, F., and von Riesen, I., *Biochem. Z.*, 1928, **203**, 22.

<sup>16</sup> Astrup, T., and Permin, P. M., *Nature*, 1947, **159**, 681.

<sup>17</sup> Goldhaber, P., Cornman, I., and Ormsbee, R. A., *Proc. Soc. Exp. Biol. and Med.*, 1947, **66**, 590.

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<sup>14</sup> Carrel, A., *J. Exp. Med.*, 1923, **38**, 407.

# 17293. Studies of Experimental Pulmonary Edema. I. Pulmonary Edema from *l*-Epinephrine and *l*-*nor*-Epinephrine (Arterenol).\*

EATON M. MACKEY AND ELDEN F. PECKA, JR.

*From the Scripps Metabolic Clinic, La Jolla, Calif.*

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**Experimental.** Fasted guinea pigs were used with equal sex distribution in the various groups. The *l*-epinephrine<sup>†</sup> and the *l*-*nor*-epinephrine (*l*-arterenol) were administered as the hydrochlorides in 0.9% NaCl in concentrations near 0.1%. In those experiments in which it was used, the adrenergic blocking agent, N-(9-fluorenyl)-N-ethyl- $\beta$ -chloroethylamine-HCl ("SKF-501")<sup>‡</sup> was dissolved

in saline as a 0.1% solution for doses of 2 mg/kg 10-15 minutes before the pressor amine. The solutions were given intravenously via the penile vein in males and intracardially in female animals. There were 6 guinea pigs in each group. The degree of pulmonary edema was determined by the weight of the lungs.<sup>3</sup>

**Results.** Our data are summarized in Table I. The *l*-epinephrine, except in the dose of .001 mM/kg (from which they recovered and were sacrificed with ether an hour later), was rapidly fatal. Groups 3 and 4 survived an average of 11 and 4 minutes respectively. On the basis of previous experience, *l*-*nor*-epinephrine was used in larger doses than the epinephrine but although the higher doses (Groups 8 and 9) prostrated the animals, they recovered and were etherized an hour after the drug was given. Higher doses of *nor*-epinephrine were required to cause pulmonary edema and with the highest dosage used, .004 mM/kg, it failed to exceed that which resulted from one-fourth as much epinephrine.

These results are in accord with those of Tainter, Tullar and Luduena<sup>11</sup> who found that the LD<sub>50</sub> of intravenously administered *l*-*nor*-epinephrine in mice is eight times less than that for *l*-epinephrine. Undoubtedly these results were in part due to pulmonary edema.

Stone and Loew,<sup>3</sup> and others have shown that certain adrenergic blocking agents are capable of reducing epinephrine-induced pulmonary edema in animals and this effect has been used as one measure of the effectiveness of adrenergic blocking agents. We administered N-(9-fluorenyl)-N-ethyl- $\beta$ -chloroethylamine ("SKF-501"), a  $\beta$ -haloalkylamine, 10-15 minutes before the pressor amines. It prevented the occurrence of any symptoms whatever or of grossly measurable pulmonary edema otherwise induced by toxic doses of either *l*-epinephrine or *l*-*nor*-epinephrine.

\* Supported by a grant from the Life Insurance Medical Research Fund.

<sup>1</sup> Meltzer, S. J., *Am. Med.*, 1904, **8**, 191.

<sup>2</sup> Emerson, H., *Arch. Int. Med.*, 1909, **3**, 368.

<sup>3</sup> Stone, C. A., and Loew, E. R., *Proc. Soc. Exp. Biol. and Med.*, 1949, **71**, 122.

<sup>4</sup> Schümann, H., *Klin. Wschr.*, 1948, **26**, 37.

<sup>5</sup> Holton, P., *Nature*, 1949, **163**, 217.

<sup>6</sup> Piteairn, D. M., and Youmans, W. B., *Fed. Proc.*, 1949, **8**, 127.

<sup>7</sup> Euler, U. S. von, and Hamberg, U., *Nature*, 1949, **163**, 642.

<sup>8</sup> Goldenberg, M., Faber, M., Alston, E. J., and Chargaff, E. C., *Science*, 1949, **109**, 534.

<sup>9</sup> Tullar, B. F., *Science*, 1949, **109**, 536.

<sup>10</sup> Bulbring, E., and Burn, J. H., *Nature*, 1949, **163**, 363.

<sup>†</sup> The *l*-epinephrine (Adrenalin) used was a specially purified sample of the natural product for which we are indebted to Dr. Leon A. Sweet of Parke, Davis & Company. It contained only a trace of *l*-*nor*-epinephrine (arterenol).

<sup>‡</sup> We are indebted to Dr. Glen E. Ulyot of the Smith, Kline & French Laboratories for generous supplies of "SKF-501."

<sup>11</sup> Tainter, M. L., Tullar, B. F., and Luduena, F. P., *Science*, 1948, **107**, 39.

TABLE I.  
Pulmonary Edema Caused by *l*-epinephrine and *l*-nor-epinephrine in Guinea Pigs.

Group No.	Dose of drug, mM/kg body wt*	Avg body wt, g	Avg lung wt g/100 g body wt	% change
Controls				
1	0	540	0.55 ± .09	—
<i>l</i> -epinephrine				
2	.0010	585	1.27 ± .14	+131
3	.0015	520	1.72 ± .13	+210
4	.0020	615	1.62 ± .06	+191
<i>l</i> -epinephrine preceded by N-(9-fluorenyl)-N-ethyl- $\beta$ -chloroethylamine HCl†				
5	.0020	605	0.55 ± .08	0
6	.0050	630	0.56 ± .08	+ 2
<i>l</i> -nor-epinephrine ( <i>l</i> -arterenol)				
7	.0020	515	0.97 ± .16	+ 76
8	.0030	480	1.13 ± .18	+105
9	.0040	505	1.25 ± .26	+127
<i>l</i> -nor-epinephrine preceded by N-(9-fluorenyl)-N-ethyl- $\beta$ -chloroethylamine HCl†				
10	.0040	550	0.51 ± .12	— 7
11	.0050	510	0.58 ± .11	+ 5

\* 1 mM epinephrine = 183 mg and 1 mM *nor*-epinephrine = 169 mg. † Adrenergic blocking agent known as "SKF-501" (see text).

**Summary.** 1. As measured by lung weight a lesser degree of pulmonary edema is produced by toxic doses of *l*-nor-epinephrine than smaller but toxic doses of *l*-epinephrine.

2. N-(9-fluorenyl)-N-ethyl- $\beta$ -chloroethylamine, a  $\beta$ -haloalkylamine adrenergic block-

ing agent, prevents all symptoms and the development of pulmonary edema due to toxic doses of either *l*-epinephrine or *l*-nor-epinephrine.

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## 17294. The Mechanism by Which Dibenamine Blocks Pituitary Activation in the Rabbit and Rat.

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(Introduced by Duncan C. Hetherington.)

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Markee, Sawyer, and Hollinshead<sup>1,2</sup> presented strongly indicative evidence that hypothalamic control of the release of luteinizing hormone from the rabbit hypophysis is exerted via a neurohumoral mechanism of which an adrenergic mediator is the final component. Ovulation, significant of LH release, was induced by injecting tiny amounts of adrenalin

directly into the adenohypophysis.<sup>2</sup> Sawyer *et al.*<sup>3,4</sup> reported confirmation of the adrenergic nature of the secretion stimulus by blocking copulation-induced ovulation with the adrenergic-blocking agent dibenamine (N,N-dibenzyl- $\beta$ -chloroethylamine).<sup>5</sup> Everett, Sawyer, and Markee<sup>6,7</sup> extended the investigation

<sup>1</sup> Markee, J. E., Sawyer, C. H., and Hollinshead, W. H., *Endocrinology*, 1946, **38**, 345.

<sup>2</sup> Markee, J. E., Sawyer, C. H., and Hollinshead, W. H., *Recent Progr. Hormone Research*, 1948, **2**, 117.

<sup>3</sup> Sawyer, C. H., Markee, J. E., and Hollinshead, W. H., *Endocrinology*, 1947, **41**, 395.

<sup>4</sup> Sawyer, C. H., Markee, J. E., and Townsend, B. F., *Endocrinology*, 1949, **44**, 18.

<sup>5</sup> Nickerson, M., and Goodman, L. S., *Fed. Proc.*, 1948, **7**, 397.

TABLE I.

Effects of Dibenamine, Priscol and 2-Dibenzylaminoethanol on Activation of LH-Release by the Anterior Hypophysis.

Species	Drug	Dose, mg/kg	Time of injection (relative to nervous stimulus)	Ovulated (hypophysis stimulated)	Failed to ovulate (stimulus blocked from reaching hypophysis)	% blocked by drug
Rabbit	Dibenamine*	25-32	<60 sec. after	3	16	84
	Priscol*	40-52	<60 sec. after	9	1	10
	2-dibenzyl-aminoethanol	25-30	<45 sec. after	9	1	10
Rat	Dibenamine†	30	0-6 hr before	8	21	72
	2-dibenzyl-aminoethanol	30	>2 hr before	5	0	0

\* Data from Sawyer *et al.*<sup>3</sup>† Data from Everett *et al.*<sup>7</sup> Six of the 8 ovulating rats were partially "blocked" as evidenced by incomplete or delayed ovulation.

to the rat, finding that, in this spontaneously ovulating species, dibenamine also blocked LH-release.

Dibenamine effectively blocked ovulation in the rabbit only if injected intravenously within 60 seconds post coitum; if delayed a few minutes, ovulation followed in all cases.<sup>3</sup> Since dibenamine does not exert its maximal adrenergic-blockade until at least 1.5 hours after injection, Nickerson<sup>8</sup> recently suggested that the drug may inhibit LH release in the rabbit by a central nervous effect independent of adrenolytic activity. Nickerson suggested further that this possibility be controlled by the use of 2-dibenzylaminoethanol, a dibenamine-hydrolysis product which retains the central excitant properties but lacks the adrenergic-blocking power of dibenamine. The present experiments demonstrate that 2-dibenzylaminoethanol does not prevent activation of LH-release in the rabbit or rat, and that at least certain anti-adrenergic properties of dibenamine are exerted within a matter of seconds after intravenous administration.

#### Sexually mature female rabbits and rats

<sup>6</sup> Sawyer, C. H., Everett, J. W., and Markee, J. E., *Endocrinology*, 1949, **44**, 218.

<sup>7</sup> Everett, J. W., Sawyer, C. H., and Markee, J. E., *Endocrinology*, 1949, **44**, 234.

<sup>8</sup> Nickerson, M., *Endocrinology*, 1949, **44**, 287.

were employed. The rabbits were mated with a vasectomized buck, and 2-dibenzylaminoethanol\* was injected intravenously during the following 45 seconds. The rats, whose vaginal-smear records had each shown two or more regular 4-day cycles, were injected with the drug intravenously at noon on the day of proestrus. Previous work<sup>7</sup> had revealed that 4-day cyclic rats of our colony receive the neurogenic stimulus for the release of LH between 2 and 4 P.M. on the afternoon of proestrus.

The central excitatory effects of 2-dibenzylaminoethanol in rabbits are more dramatic though shorter lived than those induced by dibenamine. The most commonly employed dosage of 2-dibenzylaminoethanol, 25 mg/kg, always induced severe convulsions lasting up to 20 minutes, and this dose level was nearer the LD<sub>50</sub> than was 30 mg/kg dibenamine. A dosage of 30 mg/kg 2-dibenzylaminoethanol was above the MLD for rats, but in this species the excitatory effects were somewhat less marked than those following a similar dose of dibenamine.

The results of injections of 2-dibenzylaminoethanol, as they pertain to pituitary activation are summarized in Table I and compared with the results of earlier work with dibenamine and priscol (benzyl-imidazoline).

\* Generously supplied by Dr. Nickerson.

TABLE II.  
Relative Potencies of Drugs to Counteract Immediately a 2X-Lethal Dose of Adrenalin in Rabbits.

Drug	Dose, mg/kg	Timing of drug injection in seconds from 0 time		Timing of 1 mg/kg adrenalin inj. in seconds from 0 time		Survived	Died	% protection
		Began	Ended	Began	Ended			
				0	15	0	10	0
Dibenamine	30	0	45	45	60	5	0	100
Priscol	40	0	45	45	60	1	4	20
2-Dibenzyl- aminoethanol	25	0	45	45	60	0	3	0

It is apparent that, whereas dibenamine is highly effective in blocking pituitary activation in both rabbits and rats, 2-dibenzylaminoethanol, like priscol, is quite ineffective in preventing the neurogenic stimulus from reaching the hypophysis.

A second series of experiments was designed to demonstrate whether dibenamine exerts any of its anti-adrenergic activities within the first minute following intravenous injection. The experiment is outlined and the results summarized in Table II. The rabbits receiving the intravenous adrenalin alone immediately revealed maximally dilated pupils, muscular hypotonia and respiratory embarrassment followed in a few minutes by death. When adrenalin injections were preceded within the minute by dibenamine, the subsequent symptoms were those of dibenamine only—pupillary constriction and spontaneous muscle contractions. Priscol delayed the lethal effect of adrenalin for many hours in 4 animals, and a fifth recovered. However, neither priscol nor 2-dibenzylaminoethanol counteracted the pupillary dilation induced by adrenalin. The 3 rabbits receiving 2-dibenzylaminoethanol and adrenalin expired as rapidly as with adrenalin alone. Although

protection against the lethal action of adrenalin is not a highly specific test of adrenolytic activity,<sup>9</sup> the fact that dibenamine-treated rabbits showed none of the symptoms of adrenalin poisoning mentioned above, indicates that at least part of dibenamine's adrenergic-blocking capacity becomes effective almost immediately after injection of high dosages. The results indicate that dibenamine blocks pituitary activation, not by its central excitatory effects but rather by its specific adrenergic-blocking capacity.

*Summary.* Dibenzylaminoethanol, a non-adrenolytic dibenamine-hydrolysis product which retains the central excitatory action of dibenamine, fails to block the activation of LH release from the rabbit or rat hypophysis. Dibenamine in large doses exerts at least part of its anti-adrenergic properties almost immediately after injection. The evidence indicates that dibenamine blocks pituitary activation by its specific adrenergic-blocking capacity rather than by its central excitatory action.

<sup>9</sup> Nickerson, M., Berghout, J., and Hammerstrom, R. N., *Fed. Proc.*, 1949, **8**, 322.

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## 17295. Intraventricular Pressure Curves of the Human Heart Obtained by Direct Transthoracic Puncture.\*

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In 1943, one of us (W.C.B.) studying blood pressures by the direct puncture technic had occasion to obtain a pressure curve from both the right and left ventricle by passing a needle directly into the cavity of these heart chambers. The recent development of right heart catheterization and the attempt to catheterize the left ventricle in man via the carotid artery, a procedure not without hazard, suggested to us the merits of publishing this experience with direct heart puncture. This is particularly timely because the experience with catheterization has shown not only how difficult but also how important for proper diagnosis it is to obtain adequate pressure curves. This difficulty obviously could be avoided by using the direct puncture technic as opposed to the long catheter method. Unfortunately, the hazards associated with direct puncture rule out this method for ordinary use.

The record shown in Fig. 1 was obtained on a 65 year old male who was moribund because of a cerebral thrombosis and terminal bronchopneumonia. The procedure had no significant effect upon the clinical course. A Hamilton needle manometer of adequate frequency similar to that usually employed for intra-arterial pressure recordings was used to record the intracardiac pressures. This was mounted on a portable electrocardiographic machine as previously described.<sup>1</sup> The right ventricular pressure curve (Fig. 1) was obtained by inserting the needle in the fourth intercostal space just to the left of the sternum. No electrocardiogram was recorded at this time. A respiratory fluctuation in the right ventricular pressure curve can be seen,

the minimum end diastolic pressure was 15 mm Hg and the maximum systolic pressure was 40 mm Hg. There was thus evidence of right heart congestion with little increase in systolic pressure. The contour of the curve is very satisfactory and shows none of the distorting artifacts so commonly seen with the catheter technic.

The top segment shows lead 2 of the electrocardiogram together with the left ventricular pressure curve. This was obtained by inserting the needle in the fifth intercostal space just beyond the left nipple line where the maximum apex beat was felt. In this instance there are frequent ventricular premature systoles occurring at times as pairs and triads. The third beat after the first pair of ventricular premature systoles is a fusion beat (labelled 3 on the left ventricular pressure curve). The beginning and end of the record present an acceptable undistorted left ventricular pressure curve. The pressure in this ventricle varied with respiration, showed a minimal end diastolic pressure of 15 mm Hg and a maximum systolic pressure of 100 mm Hg.

In both the left and right ventricular pressure curves, a noticeable auricular pressure wave is seen to precede the ventricular curve and is responsible to a large extent, especially in the left side, for the high diastolic end pressure. The slope of the ventricular pressure curves throughout systole and diastole is similar to that obtained by adequate pressure recordings from the cavities of the dog's heart.

The middle section of the top record offered the opportunity of studying the effect of premature systoles upon the pressure curve of the left ventricle. The beats of interest in the *middle section* have been indicated by numbers from 1 to 10. They will be referred to by these numbers in the subsequent discussion.

† This department is supported, in part, by the Michael Reese Research Foundation.

\* Aided by the A. D. Nast Fund.

<sup>1</sup> Buchbinder, W. C., and Sugarman, H., *Arch. Int. Med.*, 1940, **66**, 625.



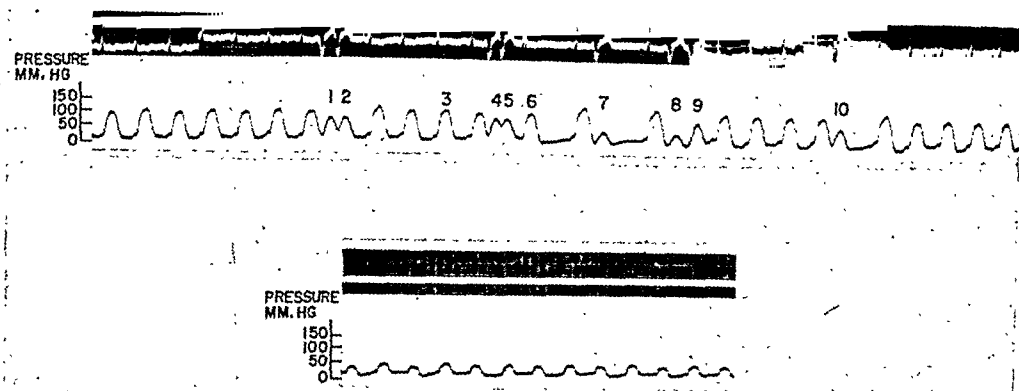


Fig. 1.

Pressure curves of left (above) and right (below) ventricle obtained in a 65-year-old moribund male. Time in each record is in the simultaneously recorded electrocardiographic record (each space between heavy lines equals 0.2 sec.). In the middle section of the upper strip, 1 to 10 indicate the successive premature systoles; 3 is a fusion beat. Discussed in text.

In accordance with Wigger's rule<sup>2</sup> the amplitude of the pressure curve of the post-extrasystolic beat is an inverse function of the duration of the immediately preceding diastole. Thus the magnitude of the pressure pulse occurring after the first pair of premature systoles (beats 1 and 2) is greater than the pressure pulse occurring before the premature systoles. This increase in size of the pressure pulse is more marked after the next two pauses (after beats 6 and 7) and after the last pause (after beat 10). Because there is no pause after the last pair of premature systoles (beats 8 and 9), the size of the post-extrasystolic beat is not significantly larger than the beats that follow. Following the last pause (after beat 10) there is a temporary mechanical alternans in that the second beat after this pause is smaller than the third. The beat registered at the time of the fusion complex (beat 3) is no different than the others. This evidence tends to confirm for man what has been established in the experimental animal that the magnitude of the pressure pulse varies with the duration of the preceding diastole.

The fact that the premature systoles occur at varying time intervals from the preceding beat accounts for the varying size and summation of their pressure curves. The shorter the R-R interval preceding the premature beat the higher up on the pressure curve of

the preceding beat does the premature beat begin. The height of the systolic pressure obtained however, is not determined exclusively by the preceding R-R interval. Thus the second and third beats in a run of premature systoles (beats 6 and 9) unexpectedly may have a higher systolic pressure than other immediately preceding premature beats in the same runs (beats 4 and 8). This might be explained by the fact that the first premature systoles are less effective in emptying the ventricular content so that the systolic residue added to the next filling makes the subsequent beat more effective. Furthermore, the duration of the contraction curve of the premature systole is shorter than the normal beat permitting more time for filling before the next premature beat. It may be, however, that this phenomenon is due to the presence of a supernormal phase expressing itself in an increased contractility of the second premature beat.

Another interesting phenomenon is revealed in the pressure curves obtained in the first (beats 1 and 2) and second (beats 4 and 5) runs of the premature systoles. These pressure curves may be said to resemble a summation curve such as is obtained with skeletal muscle. As a matter of fact the pressure during the first two premature beats of the second run (beats 4 and 5) decreases very little; in this instance, therefore, the heart has for practical purposes changed its activity

<sup>2</sup> Wiggers, C. J., *Am. J. Physiol.*, 1925, **72**, 188.

from its normal creation of pressure fluctuation intermittently to maintaining a sustained pressure rise. This suggests the possibility that a rapid run of ventricular premature systoles could leave the heart more or less in sustained systole.

*Summary.* The recording of direct pressure

curves, in which technical errors are reduced to a minimum, from the ventricular cavities of the heart in man emphasizes the great need of interpreting with care pressure curves obtained by other means in this newly expanded field of human physiology.

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## 17296. Spiral Arterial Structures in the Fetal Placenta.

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Spiraled arterial vessels are known to exist in the uterus<sup>1,2</sup> and ovary.<sup>3,4</sup> In the course of studying the fetal vascular components of the human placenta, the spiral nature of the primary branches of the allanto chorionic vessels has been noted for the first time. These appear to penetrate the substance of the placenta in much the same way as the radiate arteries do the myometrium of the uterus. The fetal spiral arteries arise from the major subchorionic vessels and represent the main vascular channels for the primary villus stem.

One hundred and twenty-five born placentae have been injected with several different masses and the vascular ramifications of the placental fetal vessels are being studied with a variety of technics. Each injection mass has been introduced via a cannulated umbilical artery shortly after the expulsion of the placenta. Fifty of these have been injected either with a 28% or 12% solution of vinyl acetate.

The more concentrated solution was injected at 200-250 mm Hg while the more dilute monomer was injected at 120 mm Hg. The placentae were corroded in commercial concentrated hydrochloric acid for 24-48 hours and in turn washed with jets of tap water. The plastic cast of the fetal vessels which was subsequently recovered represented an accurate 3 dimensional model of the vasculature. Serial reconstruction by comparison is a time consuming, laborious task which does not yield as good a result.

The spiral features can be demonstrated more easily in preparations made with the more dilute vinyl acetate injected at physiological pressures. It is our impression that the greater pressure head which is required to completely inject the more concentrated solution straightens out some of the arterial coils. The remainder of the placental preparations, comprising 3 groups of 25 each, were injected at physiological pressures with radio opaque gelatin mass, liquid latex, and India ink, respectively. Each of these masses has advantages as well as distinct limitations. In the overall study, the desirable features of each has been utilized to reconstruct the details of the vascular pattern. The gelatin and India ink injected placentae have been cleared with a modified Spalteholz technic and then selected areas have been serially sectioned for histologic detail. Liquid latex can be made to set within the vessel wall without distortion of the lumen, diameter, or calibre of the vessel. Such preparations permit a geometric

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<sup>1</sup> Hunter, Williams, *Anatoma uteri humani gravidi tabulis illustrata*, 1774.

<sup>2</sup> Daron, G. H., *Am. J. Anat.*, 1936, 58, 349.

<sup>3</sup> Reynolds, S. R. M., *Am. J. Obst. and Gynecol.*, 1947, 53, 221.

<sup>4</sup> Delson, B., Lubin, S., and Reynolds, S. R. M., *Endocrinology*, 1948, 42, 124.



Fig. 1a (top).

Spiraled fetal placental vessels. A corroded plastic preparation revealing arterial coil and partly, paid out arterial coil of primary branch of chorio allantoic vessels.  $\times 3$ .

Fig. 1b (bottom).

Magnified view of coiling and uncoiling of spiraled fetal vessel shown in area S (Fig. 1a).  $\times 25$ .

analysis and provide some insight into the hemodynamics of the placental circulation.

All of the preparations reveal aspects of spiraling. Fig. 1 is a corroded plastic cast in which an attempt has been made to isolate by dissection a single allanto-chorionic vessel

with its primary spiraled branch and terminal cotyledonary tuft. The spiraled primary villus vessel arises at a right angle to the chorionic plate from the large subchorionic vessels. It forms a helix of slowly diminishing diameter as it passes towards the decidua plate. The number of coils varies considerably averaging between 2 and 5. Some of these appear to be uncoiling and others almost completely paid out. There is a rather marked decrease in the relative diameters between the terminal spiraled segments and the delicate vascular components of the cotyledonary tuft.

*Discussion.* Physiologic considerations fail to support present concepts of morphologic adaptation of the utero-placental vasculature. Placental transfer is poorly understood, and what information is available fails to support a simple diffusion exchange.<sup>5</sup> A reinvestigation of the essential morphologic features of the fetal vasculature in the placenta has been initiated in order to reevaluate our present concepts. Spiraled arterial and arteriolar vessels in the uterus and ovary are considered to reflect a trophic response to the presence of steroid hormones.<sup>6,7</sup> The mechanism by which this particular morphologic feature of fetal placental arterial spiraling arises is not known. The human placenta is well known as a site of abundant formation of steroid hormones throughout the course of pregnancy.<sup>8</sup> The occurrence of similar spiraled vascular structures, in placental stroma, by analogy, may be attributable to a similar response.

The finding of a helical arrangement on the fetal aspect of the utero placental circulation draws attention to the spiral tuft arrangement of the maternal vessels which tap into the intervillous space. These were first described by Spanner,<sup>9</sup> who contended that this

<sup>5</sup> Barcroft, J., *Researches on Prenatal Life*, Charles C. Thomas, 1947.

<sup>6</sup> Reynolds, S. R. M., *Proc. Soc. Exp. Biol. and Med.*, 1948, **68**, 96.

<sup>7</sup> Okkels, H., and Engle, E. T., *Acta path. et microbiol. Scand.*, 1938, **15**, 150.

<sup>8</sup> Wislocki, G. B., and Bennett, H. S., *Am. J. Anat.*, 1943, **73**, 335.

<sup>9</sup> Spanner, R., *Z. f. Anatomie*, 1936, **105**, 163.

arrangement provided for a gradient of pressure which allowed for a continuous uninterrupted flow into the intervillous space. The fetal spiral arrangement suggests a similar functional adaptation which can provide for maximal fetal vascular exchange.

Based upon studies of hydrostatic conditions and maternal blood flow in rabbit uteri during pregnancy, Reynolds has elaborated a concept of uterine accommodation. In the last third of pregnancy the period of uterine growth is supplanted by a period of uterine stretching which follows upon an abrupt conversion of the conceptus from spherical to cylindrical form.<sup>10-13</sup> Ramsey has indicated that a transition is to be observed in the maternal vessels of the endometrium of the pregnant rhesus monkey beyond the 52nd gestational day. The coils of the arteries are paid out, corresponding to the period of uterine stretch-

ing.<sup>14</sup> The finding of partially uncoiled paid out spirals of placental fetal vessels in mature term placentae is consistent with these observations. The placental vessels must be subjected to the same hydrostatic conditions and mechanical stretching.

*Summary.* The spiral nature of the primary villus stem vessels has been described for the first time. The underlying mechanism suggests a trophic response to steroid hormones, comparable to the effect produced on vessels of the uterus and ovary. This spiral pattern provides a gradient of pressure which may slow fetal circulation through the placenta and allow for a more thorough exchange.

We are greatly indebted to Dr. S. R. M. Reynolds and Mr. Chester Reather of the Carnegie Institution of Washington, Baltimore, Md., for much valuable assistance. Dr. Reynolds has confirmed some of our observations and offered many helpful suggestions. Mr. Reather has provided us with excellent photographic material.

<sup>14</sup> Ramsey, E. M., Carnegie Inst. Wash. Pub. 583, "*Contributions to Embryology*," 1948, **33**, 113.

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## 17297. Effects of Intravenous Injection in Dogs of Staphylokinase and Dog Serum Fibrinolysin.\*

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Utilizing methods described in previous reports,<sup>1</sup> we have studied the *in vivo* effects of staphylokinase and dog serum lysin (fibrinolysin, plasmin, tryptase) in dogs. Normal dog serum contains the inactive precursor, *prolysin*, of an active fibrinolytic enzyme, *ly-*

*sin*, and substance(s), *antilysin*, capable of inhibiting this active lysin.<sup>1</sup> Staphylokinase,<sup>2,3</sup> a material obtained from staphylococcal culture filtrates, is capable, *in vitro*, of activating dog prolysin to lysin. It seemed of some interest to determine the effects of this material injected intravenously, as well as those of a potent fibrinolytic enzyme solution, prepared from dog serum by fractionation at 25%

\* This investigation was supported, in part, by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service.

<sup>1</sup> Lewis, J. H., and Ferguson, J. H., *Abstr. in Fed. Proc.*, 1949, **8**, 96.

<sup>†</sup> This terminology is used for brevity.

<sup>2</sup> Gerheim, E. B., Ferguson, J. H., Travis, B. L., Johnston, C. L., and Boyles, P. W., *Proc. Soc. Exp. Biol. and Med.*, 1948, **68**, 246.

<sup>3</sup> Lack, C. H., *Nature*, 1948, **161**, 559.

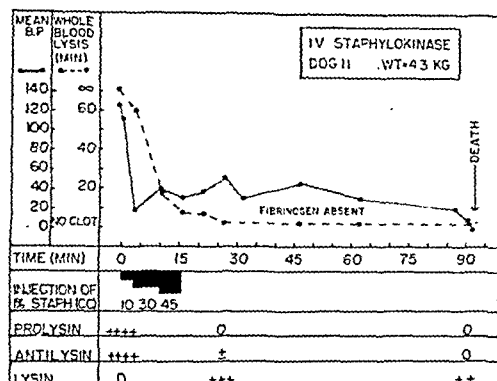


Fig. 1.

saturation with alcohol, treatment with chloroform, and aging for 6 weeks at 0°C. Due to the large amounts of these materials calculated as necessary to reproduce *in vivo* the established *in vitro* effects, we have studied only 2 dogs.

**Injection of staphylokinase.** 85 cc of a filtered 1% solution of partially purified staphylokinase was injected over a period of 16 minutes into a small fasting mongrel dog previously anesthetized with nembutal.

Fig. 1 summarizes the results observed in this dog. Arterial pressure fell precipitously following the injection of only a few cc. The injection was slowly continued and arterial pressure rose slightly, remaining between 30 and 60 mm Hg until the terminal fall. No whole blood lysis was observed in a pre-injection sample. After 10 cc of staphylokinase had been administered the whole blood lysis time was 60 minutes. As the injection continued, lysis times became shorter until at 10 minutes after the end of the injection lysis could not be measured as this blood did not clot. No significant changes were observed in whole blood coagulation times until this and subsequent specimens which did not clot even on addition of potent thrombin suggesting complete absence of fibrinogen probably caused by *in vivo* fibrinogenolysis.

Three serum samples were studied and results reported in Table I. The control specimen showed no active lysin, normal content of prolysin, as tested by *in vitro* activation with added staphylokinase, and normal antilysin titer. Serum obtained from blood taken

10 minutes after the injection showed marked lysin activity, no demonstrable prolysin (as indicated by the fact that the *in vitro* staphylokinase activation prolonged rather than shortened the lysis time), and markedly diminished antilysin. Immediately before death (90 min.) the serum still showed rather marked lysin content, no prolysin, and no appreciable antilysin. Prolysin times longer than lysin times were due to spontaneous loss of lysin which occurred during the 20 minute incubation at 37°C required for complete activation of prolysin by staphylokinase. In whole blood and serum samples obtained 10 minutes post injection and subsequently, a marked lipemia was noted. Microscopically, the globules were seen to stain readily with Sudan IV.

**Injection of dog serum lysin.** *In vitro* studies on the dog serum lysin showed it to be an extremely potent material containing approximately 4000 lysin units per cc. In order to allow sufficient lysin to combine with the calculated amount of circulating antilysin and leave a lysin excess detectable in the serum, we administered 1,000,000 units (250 cc of lysin solution) over a period of 11 minutes. Additional *in vitro* studies showed that this preparation of dog serum lysin contained a small amount of thrombin.

In an effort to minimize possible overloading of the heart due to the large volume of fluid introduced, we removed blood samples totalling 100 cc during the injection period.

Fig. 2 summarizes the observed findings in this 4.2 kg animal. Arterial pressure fell only gradually during and after the injection.

TABLE I.  
Serum Lysin, Prolysin and Antilysin after Injection of Staphylokinase.

	Lysin <sup>1</sup>	Prolysin <sup>2</sup>	Antilysin <sup>3</sup>
Control	∞	3½ min.	2460 units
10 min.	8 min.	13½ "	40 "
90 "	13 "	21 "	10 "

<sup>1</sup> Lysis time of standard clot containing 0.5 cc serum.

<sup>2</sup> Lysis time of standard clot containing 0.5 cc serum after activation with staphylokinase.

<sup>3</sup> Calculated from lysis time of standard clot containing 0.5 cc serum previously incubated with 0.5 cc standard lysin.

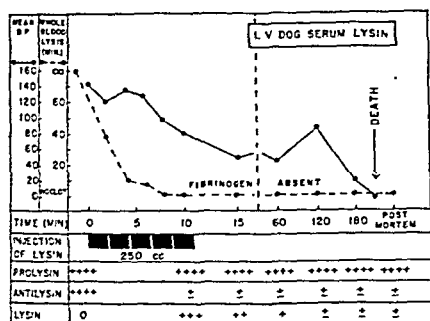


FIG. 2.

Pulse rate was noted to rise markedly during the injection. Whole blood coagulation and lysis times before, during, and following the injection are shown in Table II. The clotting times observed in the infusion period were significantly shortened at first, but after 200

TABLE II.

Whole Blood Coagulation Times and Lysis Times after Injection of Dog Serum Lysin.

	Clotting times (min.)	Lysis times (min.)
Control	7'	∞
Inj. after 50 cc	1'	39
" " 100 "	1'	8½
" " 150 "	4*	6
" " 200 "	No clot	—
" " 250 "	" "	—
Min. after inj.		
5'	No clot	—
↓	↓	—
180		—

\* Never solid.

TABLE III.

Serum Lysin, Prolysin and Antilysin after Administration of Dog Serum Lysin.

	Lysin <sup>1</sup>	Prolysin <sup>1</sup>	Antilysin <sup>1</sup>
Control	∞	3¼ min.	2300 units
After 150 cc	18 min.	3½ "	300 "
" 200 "	17½ "	3½ "	290 "
" 250 "	16 "	3¼ "	230 "
Min. after inj.			
5	24 min.	3½ min.	180 units
15	42 "	3½ "	320 "
30	90 "	3½ "	180 "
60	3-12 hr	3½ "	206 "
120	3-12 "	3½ "	252 "
180	3-12 "	3½ "	320 "
Postmortem	3-12 "	3½ "	246 "

<sup>1</sup> See footnotes Table I.

cc of lysin had been introduced no clot was formed even on addition of thrombin, indicating complete fibrinogenolysis. Clot lysis times became progressively shorter until incoagulable blood was obtained.

Table III presents the serum findings in this dog. Control serum showed no active lysin, normal prolysin and normal antilysin. During and following the injection, moderate amounts of lysin appeared and gradually diminished to a trace by one hour. Prolysin was apparently not affected as no significant changes were observed on *in vitro* activation with staphylokinase. Antilysin fell dramatically but, in spite of the presence of demonstrable active lysin, the antilysin titer never reached zero. The experiment was terminated after 3½ hours. Gross postmortem examination was performed. Blood was fluid in the heart and all large vessels examined. The heart was contracted, and, on opening, showed widespread hemorrhagic areas over the endocardium, particularly of the left ventricle. The lungs were congested and showed hemorrhagic areas in both lower lobes. Small ecchymoses were scattered over the serosal surface of the stomach. Liver and spleen appeared grossly normal. No lipemia was noted in this dog.

*Discussion.* As might be expected from known *in vitro* reactions, both staphylokinase and serum lysin, when injected, resulted in the appearance of active lysin in the withdrawn serum. Evidence of *in vivo* proteolytic activity was seen in the appearance of incoagulable blood in which no fibrinogen could be detected. The mechanisms by which these results were obtained are obviously different. Staphylokinase activated the animal's own prolysin to lysin as indicated by the fact that the prolysin titer decreased as active lysin appeared in the serum. The antilysin titer also fell, indicating that enough active lysin was formed to destroy or combine with the circulating antilysin and still leave an excess of enzyme detectable in the serum. The injection of dog serum lysin, on the other hand, apparently did not affect the circulating prolysin, but the quantity administered was sufficient to neutralize available antilysin leaving excess lysin.

No antilysin titers of zero were obtained. This is probably due to interpretation of the results produced by this method. We are unable to obtain a serum control free from antilysin and, therefore, use simply a borate buffer control. We know that lysin is a proteolytic enzyme not specific for fibrin or fibrinogen as it also attacks casein, gelatin, etc. Other proteins in serum may exert minor non-specific inhibitory effects by competing with the test substrate, fibrin.

Staphylokinase exerted a marked vasodepressor effect which may be associated with some other toxic material in the culture filtrate. Serum lysin showed lesser vasodepressor effects. Guest *et al.*<sup>4</sup> found marked vasodepressor effects following injection of their fibrinolysin<sup>5</sup> which was prepared, by a different method, from bovine serum. These authors found no changes in blood coagulation and did not report any lytic effects. It should be pointed out, however, that our experiment employed a much larger quantity of enzyme.

Some comment should be made on the marked shortening of coagulation time, from 7 minutes to one minute, immediately following administration of the first 50 cc of dog

serum lysin. Numerous experiments, in which repeated arterial sampling from a siliconed cannula has been employed to study blood coagulation times in similarly anesthetized dogs, have frequently shown wide variations between 10 and  $3\frac{1}{2}$  minutes, with a tendency to become shorter in spite of repeated saline irrigations of the cannula and discarding of the first few cc of blood at each sampling. For this reason we did not feel that the slight shortenings of clotting-time observed after injection of staphylokinase were significant. On the other hand, the marked drop in clotting-time following injection of serum lysin cannot be dismissed as an experimental error. This is possibly due to the effects of small amounts of thrombin, which were present as a contaminant of this preparation of dog serum lysin.

**Conclusions.** 1. Staphylokinase, injected intravenously into a dog, caused appearance of active serum lysin, accompanied by a fall in prolysin and antilysin. The blood became incoagulable presumably due to lysis of the circulating fibrinogen. A precipitous fall in blood pressure was also observed.

2. Dog serum lysin, injected intravenously into a dog, also resulted in the appearance of active serum lysin, fall in antilysin, but no appreciable change in prolysin. Incoagulable blood, devoid of fibrinogen, was again obtained.

Received July 13, 1949. P.S.E.B.M., 1949, 71.

<sup>4</sup> Guest, M. M., Murphy, R. C., Bodnar, S. R., Ware, A. G., and Seegers, W. H., *Am. J. Physiol.*, 1947, 150, 471.

<sup>5</sup> Loomis, E. C., George, J. C., and Ryder A., *Arch. Biochem.*, 1947, 12, 1.

## 17298. Lethal Effect of Triethylene Glycol Vapor on Air-Borne Mumps Virus and Newcastle Disease Virus.\*

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Previous laboratory investigations by Robertson and his co-workers,<sup>1,2</sup> and by Henle

\* Aided by grants from the U. S. Public Health Service, Carbide and Carbon Chemicals Corporation, and the Research Corporation.

<sup>†</sup> National Institutes of Health Postdoctorate Research Fellow.

<sup>1</sup> Robertson, O. H., Loosli, C. G., Puck, T. T., Bigg, E., and Miller, B. F., *Science*, 1941, 94, 612.

and Zellat<sup>3</sup> demonstrated that both propylene and triethylene glycol vapor were lethal for air-borne Influenza A virus. Subsequently, Rosebury and his associates<sup>4</sup> showed that triethylene glycol vapor was effective against air-borne meningopneumonitis and psittacosis

<sup>2</sup> Robertson, O. H., Puck, T. T., Lemon, H. F., Loosli, C. G., *Science*, 1943, 97, 142.

virus. These observations indicated that the glycol vapors were virucidal as well as bactericidal.<sup>2,5</sup> The experiments to be described demonstrate that triethylene glycol vapor is lethal for two more viral agents, mumps virus and Newcastle disease virus.

**Materials and methods. Viruses.** A strain of mumps virus<sup>†</sup> was cultivated in the allantoic sac of 9 day old chick embryos which, after inoculation, were incubated at 35°C for 6 days. The infected embryos were then chilled over night at 4°C and the allantoic fluid removed.

The strain of Newcastle disease virus<sup>‡</sup> was cultivated in the allantoic sac of 11 day old embryos, which after inoculation, were incubated at 37°C for 52 hours. Infected allantoic fluid was harvested after chilling overnight at 4°C.

**Virus Infectivity Titrations.** These were performed on the infected allantoic fluids which were to be sprayed into the chamber. Serial tenfold dilutions from  $10^{-5}$  to  $10^{-10}$  were made in sterile broth. A volume of 0.1 cc of the dilution of virus was inoculated through a small drill hole in the egg shell directly over the allantoic sac. Four embryos were used for each virus dilution. Each allantoic fluid was tested for its capacity to agglutinate chicken erythrocytes using the method of Salk.<sup>6</sup> Virus titration end points (E.I. 50)<sup>§</sup> were calculated by the 50% end point method of Reed and Muench.<sup>7</sup>

**The Glycol Chamber.**<sup>||</sup>, constructed of wood and glass, (Fig. 1), had a capacity of 5.5 cubic feet. The air was gently circulated by



FIG. 1.

a small fan in the left rear compartment. In front of the fan was a Screw Base Resistance Element (10 watts) which served as a source of heat to vaporize glycol impregnated paper. On the left wall of the chamber there were a series of circular coils through which ice water was circulated by means of an electrical water pump. This cooling apparatus was controlled by a thermostat set at the desired temperature.

Viral suspensions were atomized into the chamber through a port on the right side. Air bubbler samples were taken through a port on the left side. A measured volume of air, as indicated by a flowmeter,<sup>§</sup> was withdrawn into a suitable medium for trapping the infective agent. A Welch air pump was used to

<sup>2</sup> Henle, W., and Zellat, J., *Proc. Soc. Exp. Biol. and Med.*, 1941, **48**, 544.

<sup>4</sup> Rosebury, T., Meiklejohn, G., Kingsland, L. C., and Boldt, M. H., *J. Exp. Med.*, 1947, **85**, 65.

<sup>5</sup> Robertson, O. H., Bigg, E., Puck, T. T., and Miller, B. F., *J. Exp. Med.*, 1942, **75**, 593.

<sup>†</sup> We are indebted to Dr. Frank L. Horsfall, Jr., of the Rockefeller Institute for Medical Research, for the allantoic strain of mumps virus, and to Dr. Alfred S. Evans of Yale University, for the Newcastle disease virus.

<sup>6</sup> Salk, J. E., *J. Immunol.*, 1944, **49**, 87.

<sup>§</sup> 50% embryo infectivity end point.

<sup>7</sup> Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27**, 493.

<sup>||</sup> We are indebted to Dr. Charles C. Chapple of Philadelphia and to Mr. J. W. Spiezelman of Air Purification Service, Inc., for designing the Glycol Chamber.

<sup>§</sup> Lemon, H. M., and Wise, H., *Science*, 1944, **99**, 43.



No antilysin titers of zero were obtained. This is probably due to interpretation of the results produced by this method. We are unable to obtain a serum control free from antilysin and, therefore, use simply a borate buffer control. We know that lysin is a proteolytic enzyme not specific for fibrin or fibrinogen as it also attacks casein, gelatin, etc. Other proteins in serum may exert minor non-specific inhibitory effects by competing with the test substrate, fibrin.

Staphylokinase exerted a marked vasodepressor effect which may be associated with some other toxic material in the culture filtrate. Serum lysin showed lesser vasodepressor effects. Guest *et al.*<sup>4</sup> found marked vasodepressor effects following injection of their fibrinolysin<sup>5</sup> which was prepared, by a different method, from bovine serum. These authors found no changes in blood coagulation and did not report any lytic effects. It should be pointed out, however, that our experiment employed a much larger quantity of enzyme.

Some comment should be made on the marked shortening of coagulation time, from 7 minutes to one minute, immediately following administration of the first 50 cc of dog

serum lysin. Numerous experiments, in which repeated arterial sampling from a siliconed cannula has been employed to study blood coagulation times in similarly anesthetized dogs, have frequently shown wide variations between 10 and 3½ minutes, with a tendency to become shorter in spite of repeated saline irrigations of the cannula and discarding of the first few cc of blood at each sampling. For this reason we did not feel that the slight shortenings of clotting-time observed after injection of staphylokinase were significant. On the other hand, the marked drop in clotting-time following injection of serum lysin cannot be dismissed as an experimental error. This is possibly due to the effects of small amounts of thrombin, which were present as a contaminant of this preparation of dog serum lysin.

**Conclusions.** 1. Staphylokinase, injected intravenously into a dog, caused appearance of active serum lysin, accompanied by a fall in prolysin and antilysin. The blood became incoagulable presumably due to lysis of the circulating fibrinogen. A precipitous fall in blood pressure was also observed.

2. Dog serum lysin, injected intravenously into a dog, also resulted in the appearance of active serum lysin, fall in antilysin, but no appreciable change in prolysin. Incoagulable blood, devoid of fibrinogen, was again obtained.

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## 17298. Lethal Effect of Triethylene Glycol Vapor on Air-Borne Mumps Virus and Newcastle Disease Virus.\*

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Previous laboratory investigations by Robertson and his co-workers,<sup>1,2</sup> and by Henle

and Zellat<sup>3</sup> demonstrated that both propylene and triethylene glycol vapor were lethal for air-borne Influenza A virus. Subsequently, Rosebury and his associates<sup>4</sup> showed that triethylene glycol vapor was effective against air-borne meningopneumonitis and psittacosis

\* Aided by grants from the U. S. Public Health Service, Carbide and Carbon Chemicals Corporation, and the Research Corporation.

<sup>†</sup> National Institutes of Health Postdoctorate Research Fellow.

<sup>1</sup> Robertson, O. H., Loosli, C. G., Puek, T. T., Bigg, E., and Miller, B. F., *Science*, 1941, 94, 612.

<sup>2</sup> Robertson, O. H., Puek, T. T., Lemon, H. F., Loosli, C. G., *Science*, 1943, 97, 142.

<sup>4</sup> Guest, M. M., Murphy, R. C., Bodnar, S. R., Ware, A. G., and Seegers, W. H., *Am. J. Physiol.*, 1947, 150, 471.

<sup>5</sup> Loomis, E. C., George, J. C., and Ryder A., *Arch. Biochem.*, 1947, 12, 1.

TABLE I.  
Effect of Triethylene Glycol Vapor on Air-Borne Mumps Virus.

Time interval min. after spray	Control test Hemagglutination titer*					Glycol test Hemagglutination titer*				
	Individual allantoic fluids				Mean	Individual allantoic fluids				Mean
0-2	5120	5120	5120	160	2560	0	0	0	0	0
3-5	160	160	160	640	240	0	0	0	0	0
6-8	160	0	640	80	80	0	0	0	0	0
10-12	0	320	0	1280	60	0	0	0	0	0
13-15	0	1280	0	80	40	0	0	0	0	0

\* Expressed as reciprocal.

 TABLE II.  
Effect of Triethylene Glycol Vapor on Air-Borne Newcastle Disease Virus.

Time interval min. after spray	Control test Hemagglutination titer*					Glycol test Hemagglutination titer*				
	Individual allantoic fluids				Mean	Individual allantoic fluids				Mean
0-½	5120	5120	1280	5120	3840	0	0	0	0	0
1-1½	5120	5120	5120	—	5120	0	0	0	0	0
2-2½	5120	5120	1280	5120	3840	0	0	0	0	0
5-5½	5120	5120	5120	5120	5120	0	0	0	0	0
10-10½	640	0	5120	—	265	—	—	—	—	—
15-15½	0	0	0	—	0	—	—	—	—	—

\* Expressed as reciprocal.

— Not done.

cytes. This hemagglutination test was completely negative and was interpreted as indicating absence of virus rather than low titer virus.

In the first experiment with mumps virus, the agent was detected in all samples during the 15 minutes control period. In the presence of a saturated atmosphere of triethylene glycol vapor, no virus could be recovered. The chamber was then cleaned and aired for a 2 week period. When the same experiment was repeated, mumps virus could only be recovered from the first (0-2 min.) sample in the control test. It seemed possible that the minute amount of residual glycol, which had condensed on the wall of the chamber, was sufficient to interfere with the control test. There was no visible vapor and one can only guess that the percentage saturation of triethylene glycol vapor in the chamber air was very low. After heating the interior of the chamber with a 200 watt bulb to vaporize all of the residual glycol, the original experiment was easily repeated and confirmed. These observations suggest that triethylene glycol vapor is lethal for mumps virus in concentrations below saturation.

*Effect of Triethylene Glycol Vapor on Air-Borne Newcastle Disease Virus.* The virus infectivity titration of the allantoic fluid was  $10^{-8}$  E.I. 50. This material was used for a control test and a glycol test. The procedure was the same as that used in the mumps experiments with the following modifications: 1)  $1\frac{1}{2}$  cc of Newcastle disease virus was atomized into the chamber. 2)  $\frac{1}{2}$  minute air bubbler samples were taken. 3) Each bubbler contained 8 cc of 1% Casamino acids. 4) The temperature was  $74^{\circ}\text{F}$  ( $23^{\circ}\text{C}$ ) and the relative humidity was 50% during both the control and glycol tests.

*Results.* As indicated in Table II, Newcastle disease virus could be obtained from the air of the control chamber for 10 minutes after cessation of spray. No virus could be recovered when the chamber was saturated with triethylene glycol vapor.

*Discussion.* In this study, the hemagglutination test has been used as a measure of virus concentration. Beveridge and Lind<sup>12</sup> found that there was a direct relation between

<sup>12</sup> Beveridge, W. I. B., and Lind, P. E., *Australian J. Exp. Biol. and Med. Sc.*, 1946, **24**, 127.

provide positive pressure for atomization of the suspension and negative pressure for withdrawal of air through a bubbler sampler.<sup>9</sup>

The front partition of the chamber was removable and had two circular perforations 5 inches in diameter. The one on the right was sealed with a cylindrical double air lock which could permit the entry of animals of the size of guinea pigs. The one on the left was sealed by a large rubber gloved sleeve which extended into the chamber, and which could be used for exposing settling plates at periodic intervals.

Triethylene glycol was vaporized into this chamber by placing a strip of impregnated paper on the heating element. This paper contained 0.03 g of triethylene glycol per square inch. The glycol vaporized rapidly and saturated the air of the chamber.

A hygrometer consisting of a dry bulb and wet bulb was located on the back wall of the chamber. The air stream produced by the fan passed over the wet bulb and thus a fairly accurate determination of relative humidity was obtained.

*Experimental.* Preliminary experiments were conducted to determine if mumps virus could be rendered air-borne, and if so, for how long it could be recovered from the air. Infected allantoic fluid was sprayed into the chamber. Immediately after cessation of spray, serial air bubbler samples were taken for a 15 minute period. An aliquot of each sample was inoculated into the allantoic cavity of 9 day old chick embryos. After 6 days of incubation at 35°C, the fluid was harvested following a preliminary period chilling at 4°C. Each fluid was then tested for its capacity to agglutinate chicken erythrocytes. The results of this procedure indicated that mumps virus could be recovered from the air of the chamber for at least 12 minutes after cessation of spray. This was considered to be an adequate control, and the following experiments with triethylene glycol vapor were then performed.

*Effect of Triethylene Glycol Vapor on Air-Borne Mumps Virus. Control Test.* 2 cc of allantoic fluid with an E.I. 50 titer of  $10^{-8}$  was delivered by a Graeser atomizer\*\*<sup>10</sup> attached to the inlet port of the chamber. Immediately after cessation of the spray, 2 minute serial air bubbler samples were withdrawn for a 15 minute period. Each bubbler contained 10 cc of 10% horse serum in neopeptone broth. After sampling, each bubbler was treated with penicillin (500 U/cc) and streptomycin (3 mg/cc). An aliquot (0.1 cc) of each sample was inoculated intra-allantoically into 4 chick embryos. After 6 days of incubation at 35°C, the allantoic fluids were harvested. Hemagglutination titrations were then performed on the individual allantoic fluids.

*Glycol Test.* After completion of the control test the chamber was saturated with triethylene glycol vapor. The identical procedure was then repeated.

*Environmental Conditions.* The temperature was 83°F (28°C) and the relative humidity was 36% in both the control and glycol tests.

*Results.* Mumps virus was obtained from the air of the control chamber for at least 13 minutes after cessation of spray. When the air of the chamber was saturated with triethylene glycol vapor, no mumps virus could be recovered (Table I). In 2 of 4 experiments no virus could be detected even immediately after cessation of spray. In the other 2 confirmatory experiments, virus was recovered only in the first sample taken immediately after completion of spray, but at no time thereafter.

In order to determine whether negative hemagglutination represented absence of virus or low titer virus, the following procedure was carried out. The negative allantoic fluids of each sample were pooled and each pool was inoculated intra-allantoically into 4 chick embryos. After incubation and harvesting, the individual allantoic fluids were tested for their capacity to agglutinate chicken erythro-

<sup>9</sup> Lemon, H. M., *Proc. Soc. Exp. Biol. and Med.*, 1943, 54, 298.

<sup>10</sup> Prepared by Air Purification Service, Inc., Newark, N. J.

\*\* Delivers a particle size of  $1.7 \mu$  mean mass diameter.

<sup>10</sup> Graeser, J. B., and Rowe, A. H., *Am. J. Dis. Child.*, 1936, 52, 92.

TABLE I.  
 Serum Sodium/Chloride Ratios Estimated in Milliequivalents.

		A	B	C	D
Group I	Mean	1.50 $\pm$ .03	1.49 $\pm$ .02	1.41 $\pm$ .03	1.47 $\pm$ .01
Received serum only	S. Dev.	.10	.06	.08	.04
Group II	Mean	1.51 $\pm$ .03	1.39 $\pm$ .03	1.32 $\pm$ .01	1.31 $\pm$ .04
Received serum and NH <sub>4</sub> Cl	S. Dev.	.08	.09	.05	.14

A. Before any treatment was given.

B. Immediately before the second serum injection.

C. Two days after the second serum injection.

D. Immediately before termination of the experiment.

living in the animal quarters at the same time were treated in exactly similar manner except that they were given a 0.9% ammonium chloride solution instead of water to drink. The salt solution was supplied in quantities of 300 cc daily; the residue, if any, from each 24 hour period was measured. This salt solution was well tolerated, although the majority of animals gave evidence of thirst and drank most of their daily quota within a few hours of being fed. Ammonium chloride was given for one week before the first injection of horse serum and continued until the end of the experiment.

Serum sodium and chloride levels in 10 animals from each group were determined on 4 occasions: A—before any treatment was given; B—immediately before the second serum injection; C—two days after the second serum injection; D—immediately before termination of the experiment. Sodium determinations were done according to the method of Barber and Koltoff<sup>4</sup> as modified by Butler and Tuthill,<sup>5</sup> and the chloride determinations were done by the method of Wilson and Ball.<sup>6</sup>

**Results.** The means, standard deviation, and standard error of the means of the serum Na/Cl ratio expressed as milliequivalents, are shown in Table I. The serum sodium was not appreciably affected either by serum treatment or the addition of ammonium chloride to the diet or both. There was no elevation of the Na/Cl ratios of the Group I animals receiving serum alone as occurs in

rats given large amounts of D.C.A. The individual animals of this group which developed lesions and on which serum sodium and chloride determinations were made showed no elevation of the serum Na/Cl ratio. In the animals of Group 2 receiving both serum injections and ammonium chloride in their drinking water, there was a progressive elevation of the mean serum chloride, and this was reflected in a depression of the Na/Cl ratio. This was most marked following the second injection of serum, when it reached levels that were significantly lower than normal values for this group of animals before treatment or values in the Group I receiving serum alone determined at corresponding times. Five of the sodium and chloride determinations made on animals from Group 2 receiving both foreign protein and ammonium chloride were made on animals that developed arterial lesions. In two of these the Na/Cl ratio was depressed significantly below normal values.

Focal inflammatory lesions were found in the coronary and visceral arteries of both groups. The arterial changes were qualitatively similar in both groups of animals. These were comparable to the lesions produced by injections of horse serum that we have described in detail elsewhere,<sup>3</sup> and illustrated in Fig. 1. However, there was some difference in the incidence of the arterial lesions between the animals of Group 1 which received horse serum only and those of Group 2 which received both horse serum and ammonium chloride as shown in Table II. Arterial lesions were seen in 10 animals of Group 1 and in 6 of the ammonium chloride treated series. The total number of organs exhibiting arterial lesions in the animals of Group 1 was 23.

<sup>4</sup> Barber, H. H., and Kolthoff, I. M., *J. A. Chem. Soc.*, 1928, 50, 1625.

<sup>5</sup> Butler, A. M., and Tuthill, E., *J. Biol. Chem.*, 1931, 93, 171.

<sup>6</sup> Wilson, D. W., and Ball, E. G., *J. Biol. Chem.*, 1928, 79, 221.

the hemagglutination and complement fixation titers of mumps infected allantoic and amniotic fluids and concluded that the hemagglutination titer was a function of virus concentration. Ginsberg, Goebel and Horsfall<sup>11</sup> showed that the rate of increase in concentration of virus, as determined by infectivity titrations, paralleled the rise of hemagglutination titers. The latter workers also found that an E.I. 50 titer of the order of  $10^{-4.30}$  or higher was necessary before hemagglutination with mumps virus was demonstrable.

In order to rule out the possibility of a virus concentration titer too low to be detected by hemagglutination, the allantoic fluids of

<sup>11</sup> Ginsberg, H. S., Goebel, W. F., and Horsfall, F. L., Jr., *J. Exp. Med.*, 1948, **87**, 385.

the glycolized air samples were reinoculated into chick embryos. Following this second passage, no rise in titer was observed, and it was therefore concluded that no mumps virus was present.

**Summary.** 1. Under the conditions of these experiments, triethylene glycol vapor in saturated concentrations was rapidly lethal for air-borne mumps virus and Newcastle disease virus.

2. Triethylene glycol vapor was also lethal for air-borne mumps virus in concentrations below saturation.

We should like to express our indebtedness to Dr. Robert Ward for his helpful advice throughout this study.

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### 17299. Effect of Ammonium Chloride on Serum Sodium/Chloride Ratio in Foreign Serum Arteritis in Rabbits.

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An arteritis has been induced in rats by the administration of large amounts of desoxycorticosterone acetate (D.C.A.) by Selye,<sup>1</sup> and in rats so treated the development of these lesions was inhibited when  $\text{NH}_4\text{Cl}$  was added to their diet.<sup>1</sup> D.C.A. overdosage in rats also causes a rise in the serum sodium and the serum Na/Cl ratio<sup>1,2</sup> and a reduction of this ratio toward the norm following  $\text{NH}_4\text{Cl}$  therapy has been held to be significant in inhibiting the development of D.C.A. induced arteritis and other lesions in the rat.<sup>1</sup> It was thought that if this elevation of the serum Na/Cl ratio was an essential step in the pathogenesis of the D.C.A. arteritis, then if there is a common pathogenesis in the development of the D.C.A. arteritis and the well known foreign protein arteritis,<sup>3</sup> there should

also be an elevation of the serum sodium and Na/Cl ratio in animals given foreign protein which develop arteritis. It also seemed a logical possibility that ammonium chloride might inhibit the development of foreign protein arteritis if there is a common pathogenesis in the development of these two types of arteritis. The following experiments were designed to determine whether these logical possibilities concerning the pathogenesis of these two types of arteritis are correct.

**Materials and methods.** Group 1. Eighteen albino rabbits of 2-3 kg weight were given a diet of Purina Rabbit Chow and water to drink *ad libitum*. The rabbits were given an intravenous injection of sterile whole horse serum in a dose of 10 cc/kg of body weight. The serum injection was repeated after 17 days. Seven days later all animals were killed by air embolism and sections were prepared for histological study from all tissues as in our previous studies.<sup>3</sup>

Group 2. Seventeen albino rabbits of 2-3 kg weight derived from the same sources and

<sup>1</sup> Selye, H., Hall, O., and Rowley, E. M., *Lancet*, 1945, **248**, 301.

<sup>2</sup> Friedman, S. M., Polley, J. R., and Friedman, C. L., *J. Exp. Med.*, 1948, **87**, 329.

<sup>3</sup> More, R. H., and McLean, C. R., *Am. J. Path.*, 1949, **25**, 413.

TABLE II.  
Comparison of Incidence of Arteritis Between Group I and II.

Arteries	Incidence in organs		Incidence in animals	
	Group I Serum only 18 rabbits	Group II Serum and $\text{NH}_4\text{Cl}$ 17 rabbits	Group I Serum only 18 rabbits	Group II Serum and $\text{NH}_4\text{Cl}$ 17 rabbits
Coronary	10	5		
Aorta	6	2		
Mesenteric	2	2		
Gastric	1	1		
Pancreatic	4	1		
Total	23	11	10	6

compared with the incidence of lesions in the animals of Group 1 receiving horse serum alone. Whether the trend might become significant by a more effective use of ammonium chloride is not established from the results, but it seems possible for the following reason. The depression of the  $\text{Na}/\text{Cl}$  ratio did not reach significant levels until after the 24th day of the treatment and this fact may indicate that any effect of ammonium chloride on the animals as a whole was not reached until this time. It therefore seems possible that if ammonium chloride (by whatever mechanism) is responsible for the lowered incidence of lesions seen in Group 2, then more prolonged treatment with ammonium chloride before the animals receive foreign protein injections might be more effective in inhibiting the development of these lesions, and the trend toward inhibition of arteritis in the rabbits receiving both horse serum and ammonium chloride might reach significant levels. If this latter possibility were the case it might indicate a common pathogenesis for the development of the D.C.A. induced arteritis on the one hand and foreign protein induced arteritis on the other, but from what has already been said, the alteration of the  $\text{Na}/\text{Cl}$  ratio could not be an essential step

in such a common pathogenesis.

*Summary.* The possibility was considered that experimentally induced D.C.A. arteritis and foreign protein arteritis might have a common pathogenesis mediated by way of an elevation of the serum  $\text{Na}/\text{Cl}$  ratio. To test this hypothesis one group of animals was given horse serum alone, and another both horse serum and ammonium chloride. There was no elevation of the serum sodium or  $\text{Na}/\text{Cl}$  ratio in those animals receiving horse serum alone and some of the animals receiving both horse serum and ammonium chloride showed a significant depression of the  $\text{Na}/\text{Cl}$  ratio, even though they developed an arteritis.

These results clearly indicate that the development of foreign protein arteritis is not mediated through alterations of the serum sodium chloride ratio and therefore if such an alteration is essential to the development of D.C.A. induced arteritis as suggested by Selye,<sup>1</sup> then the pathogenesis of these two varieties of arteritis must be different. There was a decreased incidence of arteritis in the group of animals receiving horse serum and ammonium chloride, and it is suggested that this trend might become significant by a more effective use of ammonium chloride.

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Fig. 1.

Typical arteritis following two large intravenous injections of horse serum, presenting fibrinoid necrosis of the media with cellular exudation into the intima, media and adventitia consisting, essentially, of large mononuclear cells.

Hematin ploxine and saffron  $\times 90$ .

whereas that in the ammonium chloride treated group was only 11.

Five of the animals of Group 1 and 6 of those of Group 2 showed focal inflammatory lesions of the heart valves. Morphologically, those of the  $\text{NH}_4\text{Cl}$  treated group were indistinguishable from those of Group 1.

**Discussion.** Selye<sup>1</sup> has postulated that one important factor in the pathogenesis of D.C.A. induced arteritis is the elevation of serum sodium with consequent elevation of the serum Na/Cl ratio. In order to establish the possible relationship between the pathogenesis of D.C.A. induced arteritis and foreign serum protein arteritis, it seemed to us important to establish whether this same relationship of elevated serum Na/Cl ratios and the development of arteritis held for the production of foreign serum protein arteritis. In Group 1 receiving serum only, our results show that arteritis occurred without elevation of the serum sodium or serum Na/Cl ratio. It is clear then that if the elevation of the serum Na/Cl ratio is an essential factor in the pathogenesis of D.C.A. induced arteritis, then the pathogenesis of foreign protein arteritis

must be different. Further evidence that an elevation of the Na/Cl ratio is not important in the development of the foreign protein arteritis is indicated by the fact that in the animals receiving both horse serum and ammonium chloride two animals, at least, that developed arteritis, showed a significant reduction in the sodium chloride ratio below the mean normal value and significantly below the control pre-treatment values in these two rabbits. However, in view of the observations of Darrow and Miller<sup>7</sup> that the mineral content may vary independently in the serum and tissues, the possibility still exists that in the present experiment there may have been alterations in the sodium content of the tissues undetected by analysis of serum sodium.

The interpretation of the possible inhibitory effect of ammonium chloride on the development of the arteritis is not so clear. There was a decrease in the total number of affected animals and in the total number of organs with lesions in Group 2, but this decrease was not statistically significant when

<sup>7</sup> Darrow, D. C., and Miller, H. C., *J. Clin. Invest.*, 1942, **21**, 601.

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TABLE I.

Bromsulphalein Retention, Total Liver Lipids, Amount of Fat and Ribonucleic Acid Depletion with Standard Deviation in Normal Rats and Rats Intoxicated with CCl<sub>4</sub> with and without Previous Administration of Vitamin B<sub>12</sub>.

No. of rats	Administration of		BSP retention in mg %		Total liver lipids				Histologic fat grade		Ribonucleic acid depletion grade	
	Vit. B <sub>12</sub> , μg/100 g	CCl <sub>4</sub> , cc/100 g			g % liver		mg % body wt		Mean	S.D.	Mean	S.D.
			Mean	S.D.	Mean	S.D.	Mean	S.D.				
25			0.29	0.26								
7					4.8	1.4	166	24.2	0.15	0.28	0.65	0.75
31		0.033	2.12	0.97	8.8	1.4	366	61.6	2.00	0.70	3.60	0.87
35	15	0.033	0.90	0.60	6.2	1.4	236	41.5	0.83	0.60	2.50	0.83

ted in the distribution. Therefore the criteria used can simply be described as follows: 0 - absence of fat; 1+ - fat deposition in the central zone; 2+ - fat deposition in the central and intermediary zone; 3+ - diffuse fat deposition.

4. The ribonucleic acid depletion of the cytoplasm was graded from 0 to 5+ in methyl green-pyronine stained slides of Carnoy-fixed material. The specificity of the stain for ribonucleic acids was confirmed by digestion with ribonuclease.<sup>15</sup> Again the distribution was graded as a measure of the intensity of the depletion as follows: 0 - no depletion; 1+ - partial depletion of the central zone; 2+ - marked depletion of the central zone; 3+ - depletion of the central and intermediary zone; 4+ - depletion of the central and intermediary zone and partial depletion of the peripheral zone; 5+ - diffuse depletion.

In addition, hematoxylin-eosin stained sections were studied for confirmation.

**Results.** The rats intoxicated with CCl<sub>4</sub> showed the characteristic histologic alterations.<sup>11</sup> In comparison to the controls, there was increased deposition of lipids (demonstrated histologically and chemically), increased BSP retention and ribonucleic acid depletion. Intoxicated rats which previously had received vitamin B<sub>12</sub> showed significantly less histologic alterations than the non-treated, CCl<sub>4</sub> intoxicated rats. In the treated group, the increase in BSP retention and in fat deposition was only one-third and the ribonucleic acid depletion about one half that observed in the non-treated group. In general,

the 4 criteria studied quantitatively varied in a parallel fashion in individual animals.

**Discussion.** The previous administration of vitamin B<sub>12</sub> protects rats from CCl<sub>4</sub> intoxication to a considerable degree. This is evidenced by 4 criteria selected for quantitative studies, 2 of them histologic and 2 of them biochemical. On the average, the vitamin B<sub>12</sub> treated animals showed approximately one-third of the changes observed in non-protected rats. Although the criteria selected concerned different hepatic functions, there was marked parallelism in the degree of alterations in the individual animals, the intoxicated as well as the protected. The dose of vitamin B<sub>12</sub> used was excessively large when compared to the effective dose in anti-anemic therapy. However, the smallest effective dose in hepatic injury is yet to be determined. The dose in absolute weight is much smaller than that of any other substance used in lipotropic or protective therapy of the liver.<sup>16</sup>

The mechanism of action of vitamin B<sub>12</sub> in ameliorating the effect of CCl<sub>4</sub> intoxication upon the liver is not yet fully elucidated. Since the animals were on a balanced diet, a primary lipotropic effect, comparable to that of choline and methionine, is doubtful; the latter occurs, if at all, only in animals on low protein diet.<sup>17</sup> If, as originally assumed, the protective effect through the ribonucleic acid metabolism could be further confirmed, vitamin B<sub>12</sub> therapy would be promising in other types of hepatic injury. It is possible

<sup>16</sup> Brunshwig, A., Johnson, C., and Nichols, S., *Proc. Soc. Exp. Biol. and Med.*, 1945, **60**, 388.

<sup>17</sup> Drill, V. A., and Loomis, T. A., *J. Pharm. Exp. Ther.*, 1947, **90**, 138.

<sup>15</sup> Braehet, J., *Compt. rend. Soc. de Biol.*, 1940, **133**, 80.

# 17300. Protective Effect of Vitamin B<sub>12</sub> Upon Hepatic Injury Produced by Carbon Tetrachloride.\*

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The relation between vitamin B<sub>12</sub> and ribonucleic acid compounds is, at present, being investigated.<sup>1-4</sup> Vitamin B<sub>12</sub> has been reported to prevent the disappearance of liver basophilia which occurs when soybean oil meal is the sole source of protein in the diet of rats.<sup>5</sup> This basophilic hue of the cytoplasm of liver cells is due to the presence of ribonucleic acid compounds.<sup>6</sup> In addition, it has been shown that ribonucleic acid decreases or disappears in the initial stages of carbon tetrachloride intoxication,<sup>7,8</sup> as well as in other types of hepatic injury.<sup>9,10</sup> These findings suggested an investigation of the effect of vitamin B<sub>12</sub> upon acute hepatic injury due to CCl<sub>4</sub> intoxication in rats as an easily reproducible example of hepatic injury.

**Methods.** A total of 69 female rats, 2 to 3 months old, on a well balanced diet with a relatively high protein content received intra-

peritoneally 1 cc of mineral oil containing 0.033 cc of CCl<sub>4</sub> per 100 g of body weight. Of these, 38 received 15 µg of vitamin B<sub>12</sub><sup>†</sup> per 100 g of body weight, divided into 4 doses, injected subcutaneously 72, 48, and 24 hours prior to, and simultaneously with, the injection of the CCl<sub>4</sub>. As controls 7 normal rats of the same strain and age were used for chemical and histological examination of the liver and 25 for the bromsulphalein (BSP) retention test.

To evaluate the degree of liver damage 48 hours after the administration of the CCl<sub>4</sub> (the time of the most marked deposition of fat<sup>11,12</sup>) the following tests were done:

1. Immediately before sacrificing the animals, the serum BSP concentration was determined<sup>13</sup> in 1.5 cc of blood obtained by heart puncture 30 minutes after intraperitoneal administration of 5 mg of dye per 100 g body weight. The results were expressed in mg of BSP per 100 cc of serum.

2. The total liver lipids were determined after extraction with absolute alcohol and re-extraction of the alcoholic extract with ether according to Bloor.<sup>14</sup> The results were recorded both in grams per 100 g of liver and milligrams per 100 g of body weight.

3. The amount of fat was graded from 0 to 3+ in Sudan III stained slides of formalin-fixed material. As the fat deposition starts around the central vein, the amount is reflected

\* Supported by grants from the Dr. Jerome D. Solomon Memorial Research Foundation and the Committee on Scientific Research of the American Medical Association.

<sup>†</sup> Solomon Foundation Fellow.

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<sup>2</sup> Shive, W., Ravel, J. M., and Eakin, R. E., *J. Am. Chem. Soc.*, 1948, **70**, 2614.

<sup>3</sup> Hoffmann, C. E., Stokstad, E. L. R., Franklin, A. L., and Jukes, T. H., *J. Biol. Chem.*, 1948, **176**, 1465.

<sup>4</sup> Kitay, E., McNutt, W. S., and Snell, E. E., *J. Biol. Chem.*, 1949, **177**, 995.

<sup>5</sup> Stern, J. R., Taylor, M. W., and Russell, W. C., *Proc. Soc. Exp. Biol. and Med.*, 1949, **70**, 551.

<sup>6</sup> Dempsey, E. W., and Wislocki, G. B., *Physiol. Rev.*, 1948, **26**, 1.

<sup>7</sup> Rosin, A., and Doljanski, L., *Proc. Soc. Exp. Biol. and Med.*, 1946, **62**, 62.

<sup>8</sup> Szanto, P. B., and Popper, H., *Bull. Int. Assn. Med. Museums*, 1948, **28**, 119.

<sup>9</sup> Opie, E. L., *J. Exp. Med.*, 1946, **84**, 91.

<sup>10</sup> Szanto, P. B., and Popper, H., *Proc. Inst. Med. Chicago*, 1948, **17**, 169.

<sup>†</sup> Vitamin B<sub>12</sub> concentrate (Rubramin) in ampuls containing the equivalent of 15 µg of vitamin B<sub>12</sub> was generously supplied by E. R. Squibb & Sons, New York.

<sup>11</sup> Cameron, G. R., and Karunaratne, W. A. E., *J. Path.*, 1936, **42**, 1.

<sup>12</sup> Koch-Weser, D., *Sao Paulo Medico*, 1946, **Fevereiro**, 167.

<sup>13</sup> Casals, J., and Olitzky, P. K., *Proc. Soc. Exp. Biol. and Med.*, 1946, **63**, 353.

<sup>14</sup> Bloor, W. R., *Biochemistry of Fatty Acids*, Reinhold Publishing Corp., New York, 1943.

TABLE I.  
Effect of Nephrectomy on the Blood Pressure of Hypertensive and Normotensive Rats.

Group	Rat No.	Blood pressure in mm Hg								
		Before uninephrectomy		Total nephrectomy						
		Initial	Final	Before	5 hr	21 hr	30 hr	45 hr	55 hr	72 hr
Control	1	98	112	106	129	119	139	—	—	—
	2	112	125	118	129	122	128	—	—	—
	3	100	123	116	138	124	118	123	148	—
	4	114	115	135	128	149	—	—	—	—
	5	112	139	127	131	150	127	—	—	—
	6	105	116	105	117	124	149	126	—	—
DCA	7	94	120	166	157	158	170	154	187	—
	8	86	115	178	205	183	200	201	191	—
	9	108	107	159	173	166	158	160	187	205
	10	117	124	157	166	156	182	193	187	—
	11	96	114	152	157	164	156	166	—	—
	12	95	106	143	168	149	158	186	151	—
	13	101	117	162	171	191	156	193	193	206

Blood pressures of controls and DCA treated rats at selected intervals during the course of the experiment. Controls animals Nos. 4 and 5 proved to have a mild interstitial nephritis.

tomy.<sup>15</sup> This led the authors to conclude that hypertension of renal origin cannot be maintained in the completely nephrectomized animal. If then, hypertension resulting from DCA treatment is in reality a renal hypertension provoked by a vasculo-toxic effect of DCA on the renal vessels, an immediate return of the blood pressure from hypertensive to normal levels would be expected to occur in the totally nephrectomized DCA treated animal. This hypothesis was tested in the following manner.

**Materials and methods.** Fourteen young male albino rats weighing between 88-138 g (avg 110 g) constituted the experimental series. For a period of 2 weeks prior to the initiation of DCA treatment several determinations of blood pressure were made in order to accustom the animals to the apparatus and to determine the basal pressure of the rats. During this period they received Purina dog chow and tap water *ad libitum*. On the 15th day all animals were subjected to left nephrectomy under ether anesthesia and divided into two groups. The 8 animals of Group I received subcutaneously two compressed pellets of DCA weighing 50 mg each, while the 6 rats of Group II served as con-

trols. At this time the tap water was replaced by 0.85% NaCl solution which served as drinking fluid for both groups until the second nephrectomy was performed. The food was not altered. The sensitizing influence which uninephrectomy and sodium chloride exert on the hypertensive effect of DCA has been described previously.<sup>11</sup>

Throughout the course of the experiment blood pressure measurements were taken on the animals of both groups several times weekly until total nephrectomy, thereafter twice daily, using the method of Williams, Harrison, and Grollman<sup>16</sup> as modified by Sobin.<sup>17</sup> The measurements were made on unanesthetized animals after a preliminary warming for an adequate period in a specially constructed heating chamber, after which the tail was kept warm by the circulation of heated water through a jacket surrounding the membrane of the plethysmograph. At each determination the blood pressure was measured until two successive readings agreed to within 5 mm Hg then the 4 subsequent readings were taken and averaged. This method has proven to be simple and reliable under the conditions described.

<sup>15</sup> Rodbard, S., and Katz, L. N., *Am. J. M. Sc.*, 1939, **198**, 602.

<sup>16</sup> Williams, J. R., Harrison, T. R., and Grollman, A., *J. Clin. Invest.*, 1939, **18**, 373.

<sup>17</sup> Sobin, S. S., *Am. J. Physiol.*, 1947, **146**, 179.

that the influence upon nucleic acids is the basic function of this vitamin. This may be the mechanism of its action as the maturation factor of erythrocytes.

**Summary.** Administration of 15  $\mu$ g per 100 g body weight of vitamin B<sub>12</sub> to rats preceding acute CCl<sub>4</sub> intoxication inhibits the development of histologic changes, especially fatty metamorphosis and depletion of ribonu-

cleic acid. In addition, there is less deposition of lipids determined biochemically and less BSP retention than in the intoxicated controls. These results are tentatively related to an effect of vitamin B<sub>12</sub> on cytoplasmatic ribonucleic acid, which has been shown to disappear early in hepatic injury.

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### 17301. Persistence of Desoxycorticosterone-Induced Hypertension in the Nephrectomized Rat.

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The administration of desoxycorticosterone acetate (DCA)\* provokes hypertension characterized by persistent elevation of systolic and diastolic blood pressures. This has been observed in rats<sup>1-4</sup> and in dogs.<sup>5</sup> Elevation of blood pressure to hypertensive levels in patients with Addison's disease,<sup>6,7</sup> and also aggravation of essential hypertension<sup>8,9</sup> have been observed following DCA treatment.

Animals treated with this hormone develop nephrosclerosis<sup>1,10</sup> and periarteritis nodosa,<sup>11</sup>

vascular lesions which are frequently observed in hypertension induced experimentally by various surgical interventions on the kidney.<sup>12</sup> This raises a question as to the mechanism whereby DCA exerts its effect. The most commonly encountered views are either that DCA causes the development of hypertension which then results in widespread vascular damage, or that the vascular tissue is affected first with hypertension developing as a sequel to interference with intrarenal haemodynamics.<sup>5,13,14</sup>

Whether or not the hypertensive effect of DCA is mediated via the kidneys is a question of fundamental importance in the interpretation of experimental results involving this hormone and is also intimately concerned with the nature of hypertension accompanying adrenal cortical hyperfunction.

Hypertension resulting from the application of a constricting ligature to one renal artery subsides completely and promptly to normotensive levels following total nephrec-

\* The desoxycorticosterone acetate used in this experiment was generously provided by Dr. Erwin Schwenk of the Schering Corporation, Bloomfield, N. J.

<sup>1</sup> Selye, H., Hall, C. E., and Rowley, E. M., *Canad. Med. Assn. J.*, 1943, **49**, 88.

<sup>2</sup> Friedman, S. M., Polley, J. R., and Friedman, C. L., *J. Exp. Med.*, 1947, **85**, 187.

<sup>3</sup> Briskin, H. L., Stokes, F. R., Reed, C. I., and Mrazek, R. G., *Am. J. Physiol.*, 1943, **138**, 385.

<sup>4</sup> Green, D. M., *J. Lab. and Clin. Med.*, 1948, **33**, 853.

<sup>5</sup> Rodbard, S., and Freed, S. C., *Endocrinol.*, 1942, **30**, 365.

<sup>6</sup> McCullagh, E. P., and Ryan, E. J., *J.A.M.A.*, 1940, **114**, 2530.

<sup>7</sup> Roth, G. M., Robinson, F. J., and Wilder, R. M., *Proc. Staff Meet. Mayo Clin.*, 1943, **18**, 450.

<sup>8</sup> Goldman, M. L., and Schroeder, H. A., *Science*, 1948, **107**, 272.

<sup>9</sup> Perera, G. A., *Proc. Soc. Exp. Biol. and Med.*, 1948, **68**, 49.

<sup>10</sup> Selye, H., and Hall, C. E., *Am. Heart J.*, 1944, **27**, 338.

<sup>11</sup> Selye, H., and Pentz, I., *Canad. Med. Assn. J.*, 1943, **40**, 264.

<sup>12</sup> Smith, C. C., and Zeek, P., *Am. J. Path.*, 1947, **23**, 147.

<sup>13</sup> Selye, H., *J. Clin. Endocrinol.*, 1946, **6**, 117.

<sup>14</sup> Softer, L. J., *Diseases of the Adrenals*, 2nd edition, Lea and Febiger, 1948, 229.

clinical cases of adrenal cortical hyperfunction, especially of the Cushing's disease type. In such cases, just as in chronic essential hypertension, renal arteriosclerosis (nephrosclerosis) is usually an associated manifestation. In renal hypertension produced by the application of a partially constricting ligature to one renal artery, again hypertension and nephrosclerosis are present.<sup>20</sup> Therefore there is a school of thought which holds the hypertension of adrenal cortical hyperfunction and of essential hypertension to be due to renal mechanisms. The belief that essential hypertension is a renal disease has led to numerous attempts to relieve or cure the disease by uninephrectomy. The successes following such operations<sup>21-23</sup> have been paralleled by the failures,<sup>24-26</sup> the latter occurring even when tests revealed the remaining kidney to be normal. Thus the role of the kidney in the genesis and maintenance of essential hypertension is uncertain. However, since in animals with experimental renal hypertension removal of the ligated kidney will result, at least in the acute phase, in abolition of the hypertension, it is reasonable to assume that if DCA induces hypertension by a renal mechanism this should be "cured" by nephrectomy.

The role of the adrenal cortex, from which desoxycorticosterone may be extracted,<sup>27</sup> in essential hypertension is similarly unsettled. A high degree of correlation between the occurrence of adrenal cortical adenomas and essential hypertension has been reported by

some observers, but others have not noted the association.<sup>13</sup> Similar disagreement exists as to the status of urinary cortin in essential hypertension. The methods at present in use permit separation of the true corticoids, which may be mineral-active or carbohydrate-active, from testoid cortical hormones, but they do not sharply separate the individual hormones from one another. It may be that the cortical hormone or hormones involved in essential hypertension escape detection by current methods.

The present data indicate that the kidneys are not necessary participants in the maintenance of DCA hypertension.

This experiment does not explain the mechanism whereby DCA exerts a hypertensive effect, but it does indicate that the hormone does not induce hypertension by evoking the renin-angiotonin system. On similar grounds the hypertension of early adrenal cortical hyperactivity is probably of non-renal origin. It is possible, however, that when the nephrosclerosis develops, whether in the DCA treated animal or in the clinical conditions of essential or adrenal cortical hypertension, a renal hypertension may become superimposed upon a non-renal form.

Absence of vascular lesions in the acutely hypertensive DCA treated rat and their invariable presence in the chronically treated rat suggests that hypertension precedes nephrosclerosis in development. Therefore either hypertension causes the renal vascular lesions or the two are independent manifestations of DCA overdosage. The former hypothesis would seem to be the more tenable.

**Summary.** The blood pressure of rats rendered hypertensive by treatment with desoxycorticosterone acetate continues to rise following total nephrectomy. Hypertension of this type is not dependent upon a renal mechanism for its maintenance and it is not a consequence of renal vascular damage which is a later development. The relationship of the adrenal cortex to certain forms of clinical hypertension is discussed.

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<sup>21</sup> Platt, R., *Proc. Roy. Soc. Med.*, 1941, **35**, 317.

<sup>22</sup> Wilson, C. S., and Chamberlain, C. T., *J. Urol.*, 1942, **47**, 421.

<sup>23</sup> Semans, J. H., *Bull. Johns Hopkins Hosp.*, 1944, **75**, 184.

<sup>24</sup> Friedman, B., Moshkowitz, L., and Marrus, J., *J. Urol.*, 1942, **48**, 5.

<sup>25</sup> Weiss, E., and Chasis, H., *J.A.M.A.*, 1943, **123**, 277.

<sup>26</sup> Sabin, H. S., *J. Urol.*, 1948, **59**, 8.

<sup>27</sup> Reichstein, T., and von Euw, J., *Helvet. Chim. Acta*, 1938, **21**, 1197.

Twenty-eight days after the initiation of DCA treatment the animals of Group I had developed definite hypertension. At this time the remaining kidney was excised from animals of both groups. Inasmuch as both food and water have been shown to exert a detrimental effect on survival after total nephrectomy<sup>18</sup> both were withheld from the animals for the remainder of the experiment. Blood pressure recordings were made on each animal in the early morning and late afternoon until death supervened. The kidney which was removed at the second operation was fixed in Bouin's solution for histologic examination. One of the rats in Group I died before the second operation and was thereby eliminated from the experiment.

**Results.** The basal blood pressure values prior to uninephrectomy and the institution of DCA treatment were essentially the same in the animals of both groups. Hypertension was observed in some animals of Group I as early as the 14th day after implantation of DCA and was manifest in all of the animals of the group by the 17th day. In accordance with previous experience the animals of Group I consumed a considerably greater quantity of fluid than the controls and developed a marked diuresis. During the period before total nephrectomy the blood pressure of the control animals (see Table I) exceeded 129 mm Hg on only one occasion whereas the animals of Group I on the contrary displayed a constantly rising blood pressure. At the time of total nephrectomy the average blood pressure of the control animals was 118 mm Hg (range 105-127) while that of the treated animals was 160 mm Hg (range 143-178).

Subsequent to total nephrectomy the blood pressure of the animals in Group II remained essentially unaltered at the preoperative normotensive level (Table I and Fig. 1). The only animal in this group which survived for 55 hours had a blood pressure of 148 mm Hg. This single value is probably without significance. Single hypertensive readings were occasionally encountered in other control animals, but no definite hypertensive trend was apparent, a hypertensive reading often being

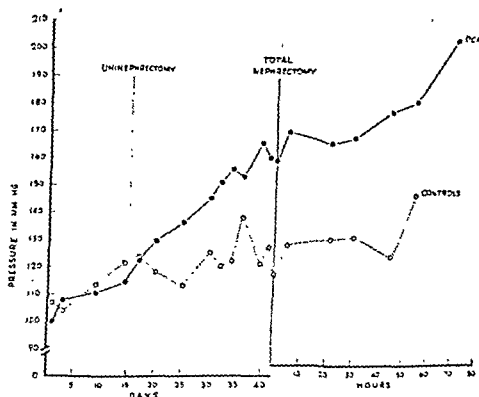


FIG. 1.

Blood pressure of DCA treated and control rats. A progressive rise in blood pressure to hypertensive levels followed the implantation of DCA on the 15th day. This was not abolished by total nephrectomy.

followed by one within the normal range. The reason for this is not clear. Further work is in progress to elucidate the reason for this behavior. Postoperatively the blood pressure of the animals of Group I rose from an average of 160 mm Hg to one of 178 mm Hg at 55 hours at which time the group was still intact, and to 206 mm Hg at 72 hours when 2 animals survived.

The results, summarized in Fig. 1, indicate that rather than declining after total nephrectomy the blood pressure of the animals of the DCA treated group continued to rise, while that of the control animals remained unaltered.

In accordance with previous findings<sup>19</sup> DCA prolonged the survival time of the completely nephrectomized animal (Table I). No attempt was made to accurately determine the time of death in these animals.

Histologic examination of the kidney which had remained *in situ* throughout the period of DCA treatment failed to reveal the presence of vascular lesions. In some of them a few convoluted tubules were dilated and an occasional cast was observed. Two of the control animals (Table I) exhibited interstitial nephritis of a mild degree.

**Discussion.** Hypertension is one of the most commonly encountered symptoms in

<sup>18</sup> Bergman, H. C., and Drury, D. R., *J. Clin. Invest.*, 1939, **18**, 777.

<sup>19</sup> Selye, H., *Canad. Med. Assn. J.*, 1940, **43**, 333.

TABLE I.  
Unsaturated Fatty Acids in the Bodies of Rats on Fat Deficient Diet and of Those Supplemented with Ethyl Linoleate.

		No. of rats	Avg wt., g	Avg lipid content/ rat, g	Linoleic*		Linolenic*		Arachidonic*	
					%	g/rat	%	g/rat	%	g/rat
Stage 1	Depletion	9	131	3.17	2.18	.068	0	0	5.76	.183
Stage 2	Appearance of symptoms	10	215	21.1	.38	.080	.37	.078	.686	.145
Stage 3	Recovery	10	276	38.4	.58	.222	.36	.138	.332	.127
Stage 3A	17 days later	5	282	40.4	.65	.252	.44	.178	.299	.121
Stage 3A + linoleate		5	323	66.6	1.71	1.139	.21	.140	.388	.258

\* As calculated by the method of Brice *et al.*<sup>2,3</sup>

amounts of linoleic and linolenic acids as the rats were maintained on the fat free diet beyond their recovery stage.

The supplementation of ethyl linoleate resulted in considerable increase in total fat and the levels of both linoleate and arachidonate present in the fat. At the same time the level of linolenate declined.

*Discussion.* It is interesting that the appearance of the essential fatty acid deficiency symptoms coincides with a sharp reduction in the levels of linoleic and arachidonic acids. The accuracy of the zero value for the linolenic acid at the first stage is doubtful. The dependent nature of the simultaneous analysis for the three acids and the high level of arachidonic could have obscured the small amounts of linolenic which might have been present. The appearance of some linolenic in the second stage, when the levels of both arachidonic and linoleic declined sharply, seems to support this doubt.

The increase in the total amounts of linoleic and linolenic at the period when the deficiency symptoms had largely disappeared (3rd stage) is further indicated in the unsupplemented group of 5 rats which were killed 17 days later. Considering the fact that the diet of the rats was devoid of these acids, these findings support the explanation suggested in the previous paper,<sup>1</sup> namely that at least a limited amount of essential fatty acids is synthesized in the body of the mature rat. At these levels the increase of linoleic and linolenic acids only is observed. It is obvious, however, from the arachidonic acid level of the group supplemented with linoleate, that the level of arachidonate also increases in the body at least when

the linoleate is administered orally or when it reaches a certain level in the tissues.

Sinclair<sup>4,5</sup> concluded that the essential fatty acids including arachidonic are probably synthesized by the rat on a fat free diet, but not to a sufficient extent to fulfill the requirements. Investigators at the Massachusetts Institute of Technology<sup>6</sup> fed rats a synthetic diet containing tri-di-deuterioiso-olein for 42 days. They analyzed the fatty acids from the carcasses and found that the arachidonic acid increased as the experiment continued, suggesting that arachidonic was synthesized by the rat. This was further supported by their findings that deuterium was present in the arachidonic acid molecule, for it indicated that the arachidonic was synthesized from the deuteriumated iso-oleic acid in the diet.

In the present study the supplemental feeding of linoleate, which raised the levels and amounts of linoleate and arachidonate in the bodies, appears to have lowered the levels of linolenate. These findings support similar observations made by Rieckehoff *et al.*<sup>7</sup> They found that the trienoic acid content of heart fatty acids decreased upon supplementation with corn oil. They also found that when the fat deficient diet was supplemented with corn oil a considerable deposition of arachidonic or tetraenoic acid took place, indicating

<sup>4</sup> Sinclair, R. G., *J. Biol. Chem.*, 1932, **96**, 103.

<sup>5</sup> Sinclair, R. G., *J. Biol. Chem.*, 1936, **114**, xciv.

<sup>6</sup> Anonymous, Report of Progress in Research III, Nutr. Biochem. Lab., Department of Food Technology, Massachusetts Institute of Technology, 1948, pp. 7-8.

<sup>7</sup> Rieckehoff, I. G., Holman, R. T., and Burr, G. O., *Arch. Biochem.*, 1949, **20**, 331.



# 17302. Relation of Fat Deficiency Symptoms to the Polyunsaturated Fatty Acid Content of the Tissues of the Mature Rat.\*

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In a previous study<sup>1</sup> on the appearance of essential fatty acid deficiency symptoms in the mature rat, only the external symptoms were considered. As one of the possible explanations for the ultimate disappearance of the scaly paws, it was suggested that synthesis of at least a limited amount of an essential fatty acid might take place within the animal itself. In order to check this possibility it was decided to examine the tissues for the amount of polyunsaturated fatty acids present at the various stages of development and disappearance of the symptoms.

**Experimental and results.** The kind of rats employed and the general procedures used in this experiment were similar to those described previously.<sup>1</sup> Fat-free diet A<sup>1</sup> was fed throughout the entire experiment. The fat soluble vitamins were suspended in propylene glycol and were fed once weekly. At the end of the depletion period, during which the intake of food was limited to approximately 5 g per rat per day, 9 out of 39 rats were killed and the entire body fat was extracted for analysis, with special care to prevent any change in the fat, e.g., a low temperature (40-50°C) was maintained in the vacuum oven during the drying of tissues, and a nitrogen ebullition tube was used in all concentration operations.

The thirty remaining depleted rats were given food *ad libitum*, and 35 days later, when all showed symptoms of fatty acid deficiency, 10 rats were killed for analysis. After 71

days on the *ad libitum* regimen, at which time most of the symptoms had largely, though not completely, disappeared, 10 more rats were killed and their fat analyzed as representative of the third stage. Two groups of 5 rats each were killed on the 88th day (stage 3A); those in one group had been supplemented with 2 drops (approximately 100 mg) of ethyl linoleate daily during the last 23 days.

The polyunsaturated constituents of the body lipids were determined by the modified method of Brice *et al.*,<sup>2,3</sup> using alkali isomerization followed by ultraviolet absorption measurements with the Beckman spectrophotometer. It should be mentioned that Brice and coworkers estimated that the probable error of the results is roughly  $\pm 10\%$  of the quantity actually present when that quantity is about 10% of the total fat; and about  $\pm 25\%$  when unsaturated acid measured constitutes only 1% of the fat analyzed. The results of the analysis which represent the average of 4 separate determinations are presented in Table I.

On the basis of these data, certain trends may be observed. At the end of the depletion period (stage 1) the total amount of fat in the body is low, but the percentages of linoleic and arachidonic acid in the fat are very high; the amount of arachidonic acid per rat is also high.

In the second stage, when the deficiency symptoms appeared, a sharp reduction in the amount of linoleic acid and arachidonic acid was noted. The linolenic, which was not found in the first stage, appeared in a small amount at this stage. It is interesting to note that with the small reduction of arachidonic acid there was a steady increase in the

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1 Barki, V. H., Nath, H., Hart, E. B., and Elvehjem, C. A., *Proc. Soc. Exp. Biol. and Med.*, 1947, **66**, 474.

2 Brice, B. A., and Swain, M. L., *J. Opt. Soc. Am.*, 1945, **35**, 532.

3 Brice, B. A., Swain, M. L., Schaeffer, B. B., and Ault, W. C., *Oil and Soap*, 1945, **22**, 219.

TABLE I.  
Blocking of Ovulation by Nembutal When Injected at a Critical Hour (2 P.M.) on the Day  
of Proestrus.

Exp. series	No. of rats	Nembutal treatment		Autopsy interval post-inj.	Results		
		Hr inj.	Dose, mg/kg		Ovulation prevented	Partial interference	Fully ovulated
I	2	2 P.M.	50	19 hr	2	0	0
	2	"	30	20 hr	2	0	0
II	11	"	30	2-4 days	11*	0	0
III	2	"	50	2-3 "	2†	0	0
	19	"	30	2-3 "	19†	0	0
IV	5	4 P.M.	50	18 hr	1	1	3
	5	"	30	18 hr	1	0	4
Totals	36	2 P.M.	30-50		36	0	0
	10	4 P.M.	30-50		2	1	7

\* Ovulation was prevented throughout by certain procedures on the second and third days. (See text).

† Ovulated before termination of experiment, but age of corpora lutea at autopsy indicates clearly that ovulation was retarded one day by the initial treatment.

employed: 30 and 50 mg/kg body weight, in single intraperitoneal injections unless otherwise specified. The concentration was 60 mg/ml in a medium containing 20% propylene glycol and 10% alcohol. The lower dose, the more generally desirable, usually produces moderate anesthesia for an hour or more, with somnolence and other signs persisting in varying degrees for several hours.

The animals were adult virgin females of the Vanderbilt substrain Va, as used in the earlier work.<sup>1,2</sup> In each rat the existence of regular 4-day cycles had been established by the vaginal smear method. In such individuals the expected ovulation time is from 1:00 to 2:30 A.M. during the night following proestrus.<sup>2</sup> 9 to 11 hours after the critical period during which neurohumoral stimulation of the hypophysis presumably occurs (see above).

At the end of each experiment the animal was killed with illuminating gas. Tubal ova were looked for in the excised ampullae compressed in physiological saline between a slide and cover slip.<sup>2</sup> The ovaries were fixed in Zenker's fluid, serially sectioned at 10  $\mu$  and stained by a modified Mallory tri-acid technic.

In all rats injected with Nembutal at 2 P.M. during proestrus, ovulation was pre-

vented from occurring at the normal time (Table I). This was proven in series I by the presence of unruptured follicles on the day after treatment. In series II the proof was less direct but no less certain. Although autopsy was delayed 2 to 4 days after the initial treatment, hypophyseal stimulation on intervening days was prevented by either atropine (at 2 P.M. on the second day) or by prolonged Nembutal sedation (repeated injections beginning at 2 P.M. each day). Persistent follicles and complete absence of recent corpora lutea in these 11 rats at the termination of experiment demonstrated that the initial brief action of Nembutal during proestrus in the 2-4 P.M. interval postponed the (potential) activation of the hypophysis for a full 24 hours. In series III various modifications of treatment on the second and third days allowed stimulation of the hypophysis on one or the other of these days, as judged from the histological appearance of the new corpora lutea. Here again, the initial treatment during proestrus had delayed stimulation for a full day.

Quite differently, in series IV, when injections were made at 4 P.M. during proestrus, little interference with gonadotrophin release occurred. The high proportion of animals fully ovulating overnight compares with that

<sup>2</sup> Everett, J. W., *Endocrinology*, 1945, 43, 359.

to them that this acid could be synthesized from linoleate. Nunn and Smedley-Maclean<sup>8</sup> also observed that supplementation with linoleic or with linolenic acids resulted in the production of arachidonate.

While this work was in progress, Rieckhoff, Holman and Burr<sup>7</sup> reported on the effect of dietary fat on polyethenoid fatty acids of rat tissues. One of their conclusions was that the deposition of polyunsaturated fatty acids takes place primarily in the phospholipid fraction with very little change in the neutral fat. They also found that the effect of the diet on the occurrence of the unsaturated fatty acids is considerably greater in some organs than in others, and that the polyunsaturated acids are particularly low in the skin and depot fat. Smedley-Maclean and Nunn<sup>9</sup> also found small amounts of arachidonate in the phospholipids of liver and muscle of fat deficient rats, but none in their neutral fat.

On the basis of these observations it becomes evident that the phospholipid fraction from the various organs (especially the heart, liver, brain and kidney) should be isolated and analyzed separately for better evaluation of the changes which take place in the essen-

tial fatty acid content of the rat. Otherwise, the small amounts of these acids are diluted in the relatively large quantities of fat extracted from the entire carcass, and the changes become less evident.

**Summary.** 1. Caloric restriction, causing severe emaciation, followed by *ad libitum* feeding on the same fat-deficient diet, precipitated symptoms of fat-deficiency in mature rats. When the rats, after depletion, were maintained on the fat-free diet *ad libitum* for sufficiently long periods, spontaneous recovery was observed.

2. The tissues of these rats were analyzed for linoleic, linolenic and arachidonic acids by the spectrophotometric method. The concentrations of linoleic and arachidonic acids in the body fat were found to vary with the appearance of the symptoms; it was high at the end of the depletion period, and low at the stage when fat deficiency symptoms were present. The higher levels of linoleic and linolenic acids at the time of recovery, after a long period on the fat-free diet, are considered as further evidence for the synthesis of essential fatty acids in the mature rat.

3. Supplementation of ethyl linoleate increased the level of arachidonate in the body fat.

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<sup>8</sup> Nunn, L. C. A., and Smedley-Maclean, I., *Biochem. J.*, 1938, **32**, 2178.

<sup>9</sup> Smedley-Maclean, I., and Nunn, L. C. A., *Biochem. J.*, 1940, **34**, 884.

### 17303. The Blocking Effect of Nembutal on the Ovulatory Discharge of Gonadotrophin in the Cyclic Rat.

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(Introduced by Duncan C. Hetherington.)

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In a preceding investigation<sup>1</sup> it was indicated that in 4-day cyclic rats of the Vanderbilt strain, under our colony conditions, neurohumoral stimulation of the hypophysis occurs with great uniformity on the day of proestrus between 2 P.M. and 4 P.M., inciting ovulatory discharge of luteinizing

hormone. Administration of intravenous dibenamine (*N,N*-dibenzyl- $\beta$ -chloroethyl amine) or subcutaneous atropine at 2 P.M. or earlier characteristically blocks ovulation. Injection of either agent at 4 P.M. or later, however, rarely interferes. We have now found essentially identical time relationships with Nembutal (pentobarbital sodium).

Two dose levels of the barbiturate were

<sup>1</sup> Everett, J. W., Sawyer, C. H., and Markee, J. E., *Endocrinology*, 1949, **44**, 234.

TABLE I.  
Lethal Effect of Different Strains of *B. abortus* Injected Intra-abdominally Into Mice Together with 0.5 cc of a 10% Mucin Suspension.

Strain	Quantity injected (cc)	No. of animals			Cumulative numbers		Mortality, %
		Under experiment	Died	Survived	Died	Survived	
Local strain	0.5	8	5	3	8	3	72.7
	0.2	8	1	7	3	10	23.1
	0.1	8	2	6	2	16	11.1
	0.05	8	0	8	0	24	0
Swiss strain	0.5	8	7	1	13	1	92.9
	0.2	8	4	4	6	5	54.5
	0.1	8	2	6	2	11	15.4
	0.05	5	0	5	0	16	0
Abortus 19	0.5	8	4	4	8	4	66.7
	0.2	8	4	4	4	8	33.3
	0.1	8	0	8	0	16	0

of vaccines as well as the protective and therapeutic action of sera and chemotherapeutic substances.

**Test organisms.** Two strains, one of *B. melitensis* and another of *B. abortus*, isolated in this country were employed. In addition to these, 3 laboratory strains of *B. abortus* were employed. One was obtained from the National Type Collection in London and 2 others representing the attenuated "Abortus 19" from the Government Veterinary Laboratories in Tel Aviv. One of these strains, designated as "Swiss strain 19" proved to be richer in S-forms than the other.

**Mucin.** Mucin in scales manufactured by Burroughs Wellcome, London, was employed. A weighed amount was thoroughly ground in a mortar with glass sand, suspended in saline to give the required concentration and autoclaved. Concentrations of 5.0%, 7.5%, and 10.0% were employed.

**Experimental injections.** White mice from the laboratory stock served as experimental animals. The mice were 5 weeks old at the time of the tests and averaged 20 g in weight. Preliminary experiments with 48-hour broth cultures showed that 0.1-0.5 cc of these cultures given intra-abdominally did not produce lethal infections, though after sacrificing the animals 5 days after injection, pure cultures of *Brucellae* could be obtained from most organs. The spleens of these animals showed marked enlargement. Another group of animals now received varying quantities of 48-

hour broth cultures ranging from 0.005 to 0.5 cc, to which 0.5 cc of mucin in varying concentrations was added. These experiments which were carried out with *B. abortus* as well as with *B. melitensis* showed that a mucin concentration of at least 10% must be employed in order to obtain a lethal effect; 0.5 cc of broth culture produced a 100% mortality and 0.2 cc of broth culture a 50% mortality when injected together with 0.5 cc of a 10% mucin suspension. Mucin suspensions of 5.0% and 7.5% given with the same quantities of broth cultures were not able to produce a lethal effect. No differences were observed between *B. melitensis* and *B. abortus*.

We now examined the lethal effect of different strains of *B. abortus* given under the same conditions. The results of these experiments are summarized in Table I. While 2 of the strains, the local strain and the "Abortus" 19 proved to be relatively avirulent, both exhibiting their LD<sub>50</sub> at 0.35 cc, the Swiss strain proved to be more virulent for mice, if injected along with mucin, having a LD<sub>50</sub> at 0.19 cc.

The mice which succumbed to the infection yielded pure cultures of the infective strains from their organs. Death generally occurred after 1-2 days, though with small doses such as 0.1 cc it sometimes occurred after 4-8 days. The shorter the period between the injection and death the less was the spleen enlarged.

The surviving animals of the different ex-

found after other blocking agents at this hour.<sup>1</sup>

The blocking capacity of Nembutal *per se*, in single dose (30 mg/kg), can endure through most of 4 hours at least, since 2 proestrous rats injected at noon were "blocked." The effect does not last much longer, however, for 2 proestrous rats injected at 10 A.M. ovulated during the following night. In control experiments, 4 proestrous rats were injected intraperitoneally at 2 P.M. with 0.1 ml of 20% propylene glycol in 10% alcohol. All ovulated overnight.

The fact that Nembutal will prevent ovulation when administered at appropriate hours, furnishes additional strong evidence that neurogenic stimulation of the hypophysis is essential for ovulatory discharge of LH. Postponement of hypophyseal stimulation for a full 24 hours following the brief action of Nembutal during the 2-4 P.M. interval, implies a 24-hour periodicity in some neural (presumably hypothalamic) center which constitutes a part of the LH-release apparatus in this species. Evidence of similar nature was obtained recently in a quite different experiment, based on the 24-hour advancement of ovulation time by progesterone in 5-day cyclic rats.<sup>3,4</sup>

It is theoretically possible to block ovulation day after day by treatment with barbiturates at appropriate hours. We have, in fact, accomplished this in several cases (series II), but the question of precise timing on days following proestrus requires further analysis. Whenever we succeeded, the vaginal smears gave evidence of continued estrogen secretion and, therefore, of continued secretion of gonadotrophin at subovulatory level. This recalls the report of Westman<sup>5</sup> that certain rats were in constant estrus during 21 days of treatment with twice-daily subcutaneous 1-methyl-5,5-ethylphenyl barbituric acid.

*Summary.* Brief action of Nembutal during certain critical hours on the day of proestrus delays the ovulatory discharge of hypophyseal gonadotrophin for 24 hours. This is evidence of a 24-hour periodicity in the neural mechanism which incites such discharge.

<sup>3</sup> Everett, J. W., *Anat. Rec.*, 1949, **103**, 448.

<sup>4</sup> Everett, J. W., and Sawyer, C. H., unpublished.

<sup>5</sup> Westman, A., *Acta Med. Scand.*, 1947, **128**, Suppl. 196, 111.

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### 17304. Lethal *Brucella* Infections in White Mice Produced with the Aid of the Mucin Technic.

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Although the mucin technic has been employed in order to lower resistance against many microorganisms, no attempts have hitherto been made to produce lethal infections with *Brucellae* by means of this technic. It was found that the experimental infections produced in mice without the use of the mucin technic are mild, following a retrogressive and chronic course, so that the mouse may be used as a reservoir for keeping these pathogenic organisms alive. Lethal infections of mice have so far not been reported.<sup>1,2</sup> The

following experiments were undertaken in order to produce more acute or even lethal infections with the aid of the mucin method. We expected that the fatal outcome of such infections would enable us to determine the virulence of *Brucellae*, the immunizing power

<sup>1</sup> Lustig, A., and Vernoni, G., in Kelle, Kraus, Uhlenhuth, *Handbuch der pathogenen Mikroorganismen*, 1928, **4**, 511.

<sup>2</sup> Topley and Wilson's *Principles of Immunity*, revised by G. S. Wilson and A. A. Miles, 3rd edition, London, E. Arnolds & Co., 1948, **1**, 528.

These findings have been confirmed with respect to pneumococcal and streptococcal infections by Knight and colleagues<sup>5,6</sup> and with respect to protection against *Streptococcus hemolyticus* by Hobby.<sup>11</sup> On the other hand, in spite of *in vitro* potency, subtilin has been found ineffective against syphilitic infections in rabbits<sup>12</sup> and against *M. tuberculosis* infections in hamsters<sup>13</sup> and in mice.<sup>5,6,14</sup> Steenken and Wolinsky<sup>15</sup> found subtilin without effect on tuberculosis infections in guinea pigs, whereas Salle and Jann<sup>16</sup> have reported both positive and negative results with different lots of subtilin. Farber, Eagle, Anderson, and Gorman<sup>17</sup> found suggestive evidence of therapeutic activity from the topical application of subtilin on tuberculous laryngeal lesions.

The present work was undertaken to determine the absorption of subtilin and of methyl esters of subtilin<sup>18</sup> when administered by various routes and to note any pharmacological effects, as an indication of the value and the feasibility of more extensive clinical trials. The activity of these esters against *M. tuberculosis in vitro* is at least as high as that of subtilin.<sup>19</sup>

**Methods.** Thirty-one rabbits were used in 44 experiments. The animals used more than once had shown little or no absorption at the time of the original experiment and had not

been used for at least a month. Except for treatments by slow infusion or injection into the ligated colon, the animals were not anesthetized. Anesthesia, when used, was with sodium pentobarbital. Initially, 40 mg/kg intravenously was used, with 10 mg/kg supplements as needed to maintain a light anesthesia. Intravenous injections of subtilin were made into the marginal ear vein, subcutaneous injections were divided between the two sides of the animal so as to insure a larger surface for absorption. Intramuscular injections were divided likewise and were made into the muscles of the thigh.

The subtilin was in nearly all instances from one composited batch of highly potent material prepared by methods described elsewhere.<sup>1,2</sup> Although electrophoretic analysis, fractional dialysis and salt fractionation<sup>2</sup> indicated that our subtilin was homogeneous, investigations at the research laboratories of Merck & Co., Rahway, N. J., by counter-current distribution showed that our samples were probably about 90% pure. The methyl esters were prepared<sup>18</sup> from another batch of equally pure subtilin. Two lots were used: the first (No. 17ME-242) contained 5.9 equivalents of methoxyl per 10<sup>4</sup> g and was characterized by markedly enhanced bacteriostatic potency and 4-fold increase in solubility under physiological conditions. The second (No. 37ME-242) contained 11.9 equivalents of methoxyl per 10<sup>4</sup> g and was characterized by substantially unchanged bacteriostatic potency but 30-fold increase in solubility under physiological conditions. For intravenous administration, the solvent was 5% glucose; when 10% subtilin solutions were used for administration by other routes, the subtilin was dissolved in distilled water. Blood samples for subtilin assay were obtained by cardiac puncture. Urine, when desired, was obtained by catheter with washing of the bladder so as to assure complete collection.

Subtilin and the methyl esters of subtilin were determined by the cup-plate method. The test organisms used previously<sup>20</sup> for the

<sup>11</sup> Hobby, G., personal communication.

<sup>12</sup> Eagle, H., Musselman, A. D., and Fleischman, R., *J. Bact.*, 1948, 55, 347.

<sup>13</sup> Anderson, H. H., and Wong, S. C., *Tubercuology*, 1946, 8, 77.

<sup>14</sup> Rake, G. W., personal communication.

<sup>15</sup> Steenken, W., Jr., and Wolinsky, E., *J. Bact.*, 1949, 57, 453.

<sup>16</sup> Salle, A. J., and Jann, G. J., Presentation at the Second National Symposium on Recent Advances in Antibiotics Research, Washington, D. C., April 11-12, 1949, under the auspices of the Antibiotics Study Section of the National Institute of Health.

<sup>17</sup> Farber, S. M., Eagle, H. R., Anderson, H. H., and Gorman, R. D., *J. Lab. and Clin. Med.*, 1948, 33, 799.

<sup>18</sup> Carson, J. F., Jansen, E. F., and Lewis, J. C., *J. Am. Chem. Soc.*, in press.

<sup>19</sup> Chin, Y. C., *Fed. Proc.*, 1948, 7, 211, and unpublished data.

<sup>20</sup> Lewis, J. C., Humphreys, E. M., Thompson, P. A., Dimick, K. P., Benedict, R. G., Langlykke, A. F., and Lightbody, H. D., *Arch. Biochem.*, 1947, 14, 437.

periments were killed within a period ranging from the sixth to the 50th day after injection. The organs of the infected animals were cultured on agar plates as well as in broth tubes. In many cases the agar cultures yielded negative results, while after incubation of the broth cultures for 7 days positive results were obtained. The microorganisms isolated from these cultures were identified as *Brucellae* by agglutination with specific antisera. The following organs were examined: heart, lungs, liver, spleen, kidneys and genital glands. Seventy-six mice which were dissected up to the 28th day after injection proved to harbor *Brucellae* in all or in several organs. Fifteen out of 20 mice which were dissected between the 30th and the 50th day after the onset of the infection proved to harbor *Brucellae*, while only 5 yielded sterile cultures. The organs of 145 mice were cultured after death took place or when the mice were sacrificed. The positive results obtained from the different organs were as follows: Heart 131,

lungs 126, liver 133, spleen 136, kidneys 136, genital organs 127. These figures prove that significant differences in the infection rate of the different organs do not exist, but only that foci of infection are equally distributed over the different organ systems of the infected animal.

*Summary.* *B. melitensis* and *B. abortus* did not produce lethal infections in mice even when 0.5 cc of broth cultures were injected intraabdominally. On the other hand lethal infections were produced if the broth cultures were administered together with 0.5 cc of a 10% mucin suspension. The LD<sub>50</sub> for *B. melitensis* was 0.2 cc, for 2 strains of *B. abortus* 0.35 cc, and for a third strain of *B. abortus* 0.2 cc. In most of the surviving animals chronic infections were noted, if the dissections were performed up to the 50th day after the onset of the infection.

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### 17305. Absorption of Subtilin in the Rabbit.

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Subtilin,<sup>1,2</sup> an antibiotic active *in vitro* against Gram-positive bacteria<sup>3</sup> and *M. tuberculosis*,<sup>3-6</sup> has also been found active *in vivo* against the more sensitive pathogenic

bacteria. For example, Salle and Jann injected subtilin intraperitoneally into mice infected with pneumococcus Type III,<sup>7</sup> *Streptococcus pyogenes*,<sup>8</sup> or *Staphylococcus aureus*,<sup>9</sup> and into guinea pigs subjected to experimental anthrax infections.<sup>10</sup> Prophylactic and therapeutic effects, depending on the relations of infection and dosage times, were striking.

\* Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.

<sup>1</sup> Garibaldi, J. A., and Feeney, R. E., *Ind. Eng. Chem.*, 1949, **41**, 432.

<sup>2</sup> Fevold, H. L., Dimick, K. P., and Klose, A. A., *Arch. Biochem.*, 1948, **18**, 27.

<sup>3</sup> Salle, A. J., and Jann, G. J., *Proc. Soc. Exp. Biol. and Med.*, 1945, **60**, 60.

<sup>4</sup> Wong, S. C., Hambly, A. S., Jr., and Anderson, H. H., *J. Lab. Clin. Med.*, 1947, **32**, 837.

<sup>5</sup> Knight, V., Shultz, S., and DuBois, R., *Proc. 48th General Meeting Soc. Am. Bact.*, Minneapolis, Minn., May, 1948, p. 84.

<sup>6</sup> Knight, V., and Tompsett, R., *J. Clin. Invest.*, 1948, **27**, 544.

<sup>7</sup> Salle, A. J., and Jann, G. J., *Proc. Soc. Exp. Biol. and Med.*, 1946, **62**, 40.

<sup>8</sup> Salle, A. J., and Jann, G. J., *Proc. Soc. Exp. Biol. and Med.*, 1946, **63**, 519.

<sup>9</sup> Salle, A. J., Presentation at the Conference on Antibiotic Research, Washington, D. C., January 31 and February 1, 1947, under the auspices of the Antibiotics Study Section of the National Institute of Health.

<sup>10</sup> Salle, A. J., and Jann, G. J., *Proc. Soc. Exp. Biol. and Med.*, 1946, **63**, 41.

instance and 4 mg in another; however, absorption was so slow that 6 ppm of subtilin was the maximum found in the blood.

The preceding experiments have indicated that little subtilin gets into the blood stream unless it is placed there directly. In the case of a single injection, there is some danger and activity is short-lived. A slow, intravenous infusion might give satisfactory blood levels without seriously embarrassing the animal. Three anesthetized rabbits were used, with infusion into the jugular or femoral veins of 0.25% subtilin in 5% glucose. One animal received 20 mg/kg/hr for 2 hours, 60 mg/kg/hr for 1 hour and 150 mg/kg/hr for 30 minutes until death. At the end of the third hour the blood level was 450 ppm and 6% of the injected subtilin was found in the urine. No symptoms were observed until sometime after the rate was raised to 60 mg, when tremors in the extremities were seen. Death was apparently of cardiac origin. The second animal was given 20 mg/kg/hr for 4 hours. No symptoms were noted, the animal appearing in good condition at the end of this time. The blood level had risen to 700 ppm.

In these 2 rabbits, the blood was observed for agglutination. We had found previously that there was an *in vitro* clumping of red cells at concentrations of subtilin lower than found in these two animals. No clumping was noted, indicating that *in vivo* actions differed from *in vitro*, or that agglutinated cells had been filtered out in the capillary systems of the body.

The third rabbit receiving intravenous infusion was prepared for records of blood pressure and respiration. Approximately 20 mg/kg/hr for 1½ hours did not modify blood pressure, heart rate or respiratory rate. Raising the infusion rate to 70 mg/kg/hr caused a gradual fall in pulse pressure (as measured with a membrane manometer) and a gradual increase in respiratory rate.

Chin<sup>23</sup> has reported that the LD<sub>50</sub> of intravenously injected subtilin to mice is 100 mg/kg. This is slightly higher than the approximately 70 mg/kg found by us in a small series of animals, but either value would

give a body concentration well above the minimum *in vitro* effective tuberculostatic dose. Death from intravenously administered subtilin is presumably due to embolism since subtilin, which dissolves readily in water, is only slightly soluble in physiological saline<sup>18</sup> or serum.<sup>24</sup> The amount held in solution varies somewhat with the manner of manipulation, being around 0.05 to 0.1 g/100 cc under physiological conditions of temperature and salt concentration. The intraperitoneal LD<sub>50</sub> was some 2 to 3 times the intravenous LD<sub>50</sub>. Subcutaneously, mice were able to tolerate more than 3 g/kg of purified subtilin without demonstrable symptoms.<sup>25</sup> The lack of subcutaneous toxicity is likewise explained by the low solubility in body fluids, a deposit of precipitated material being formed at the site of injection.

**Subtilin methyl esters.** The increased solubility of some of the methylated subtilin preparations, and the increased antibiotic activity of others<sup>18</sup> led to the hope that satisfactory blood levels could be obtained. Twelve animals were used. On 4 of them blood assays were not made. There were no untoward reactions in these or any of the other rabbits receiving the esters. It was found that blood levels of the 8 other animals were slightly higher after intravenous, subcutaneous and intramuscular injection of the more soluble ester than the levels found after administration of unmodified subtilin. The levels (up to 2.4 ppm after intramuscular administration) were not great enough to warrant further study. The ester with increased activity was absorbed so poorly that it had an unimportant antibiotic effect in the blood.

Several lots of methyl esters of subtilin were tested for approximate intravenous toxicity in mice. The values obtained did not differ markedly from values for unchanged subtilin.

**Subtilin-pectin complex.** Subtilin, when dissolved with 5 to 8 times its weight of pectin gives a product which is soluble in physiological saline to the extent of 0.5% of subtilin. After some time the complex begins to

<sup>24</sup> Klose, A. A., unpublished data.

<sup>25</sup> Wilson, R. H., Lewis, J. C., and Humphreys, E. M., *Fed. Proc.*, 1948, 7, 266.

<sup>23</sup> Chin, Y. C., *Fed. Proc.*, 1947, 6, 317.



turbidimetric assay of subtilin were much too insensitive by the cup-plate technic. This insensitivity proved particularly troublesome for the methyl esters of subtilin, which had much lower potencies than subtilin by cup-plate or agar-streak methods in contrast to their behavior in broth. The cause of this anomalous behavior is not known. A bacterium tentatively identified as *Sarcina lutea* was used as the test organism for subtilin. The test medium was that previously used<sup>20</sup> for the turbidimetric assay of subtilin with *Micrococcus conglomeratus*. Incubation for 20 hours at 35° gave an approximately 20-mm zone of inhibition when a solution containing 1 ppm of subtilin was placed in the cup. An unidentified Gram-negative diplococcus (C-7) isolated from chicken feces was chosen as the most sensitive organism available for the assay of methyl esters of subtilin. Two ppm of either ester gave an approximately 20-mm zone on nutrient agar containing 5% NaCl after 22 to 48 hours' incubation at 35°C.

In all assays the antibiotic under test was dissolved in an aliquot of whole citrated rabbit blood to provide a standard response. Control blood samples taken from animals never treated previously gave no inhibition of the test organisms. Citrated blood samples from treated animals and aqueous dilutions of this blood were pipetted directly into assay cups. Dilutions were not necessary for blood samples from animals treated with the subtilin esters. All assay results are expressed in terms of the weight of the particular type of subtilin under test, since the relative potencies of the three materials varied markedly with the experimental conditions.

**Results. Subtilin.** Intravenous injection gave a satisfactory blood level, but the level was transitory and the procedure was accompanied by some danger. In a single experiment, 50 mg/kg caused death in 2 minutes, and 10 mg/kg did likewise in 1 of 5 trials. The concentration of subtilin in the blood of the other 4 animals was 100-200 ppm 5 minutes after the injection, falling to 10-30 ppm in 2 hours and to zero in 24 hours. Up to 7% of the injected subtilin was found in the 2-hour urine.

Subcutaneous and intramuscular injection of

100 mg/kg can be considered together. Blood levels ranging from a trace up to 1.4 ppm were found. A persistent lump was present at the injection site after subcutaneous injections, and a sterile abscess was present in muscles 2 months after subtilin administration. There was no antibiotic activity in an extract of this abscess, although an assay of a comparable muscle site 24 hours after injection indicated that the granular material still had considerable activity.

Intraperitoneal injection of 10 to 100 mg/kg led to blood levels of 0 to 1 ppm of subtilin. After 10 mg/kg there was no visible precipitate in the abdomen, but 50 mg/kg was sufficient to produce such a deposit.

Treatment of subtilin by crystalline pepsin or trypsin reduces its antibiotic activity.<sup>21,22</sup> This fact, along with the known low solubility of subtilin in body fluids, would suggest that administration by mouth would be ineffective. Nevertheless, 1 g/kg was given to one rabbit *per os*. The highest blood level noted was 0.1 ppm. After 5 hours, of the 4 grams originally given, only 2 µg were found in the urine, 15 mg in the intestine, and 0.5 g in the stomach. Two anesthetized animals were given 100 mg of subtilin/kg per rectum, with ligation of the gut near the anus to prevent loss. The solution was 1% of subtilin in water, and the volume was great enough to spread throughout the colon. From 2 to 4 ppm of subtilin were found in the blood stream, and autopsy 2 hours after administration showed subtilin deposits in the colon.

It is conceivable that a slow subcutaneous infusion of a dilute solution would allow the subtilin to be absorbed rather than precipitated. Concentrations of 0.05 to 0.5% in 5% glucose were infused in 3 unanesthetized animals under the skin of the back at rates of 1.7 to 27 mg/kg/hr for 6 hr. The volume of solution was approximately 5 cc/kg/hr. Some absorption did occur, as 27 mg of subtilin was found in the 24-hour urine sample in one

<sup>21</sup> Stansly, P. G., and Ananenkov, N. H., *Arch. Biochem.*, 1947, 15, 473.

<sup>22</sup> Dimick, K. P., Alderton, G., Lewis, J. C., Lightbody, H. D., and Fervold, H. L., *Arch. Biochem.*, 1947, 15, 1.

instance and 4 mg in another; however, absorption was so slow that 6 ppm of subtilin was the maximum found in the blood.

The preceding experiments have indicated that little subtilin gets into the blood stream unless it is placed there directly. In the case of a single injection, there is some danger and activity is short-lived. A slow, intravenous infusion might give satisfactory blood levels without seriously embarrassing the animal. Three anesthetized rabbits were used, with infusion into the jugular or femoral veins of 0.25% subtilin in 5% glucose. One animal received 20 mg/kg/hr for 2 hours, 60 mg/kg/hr for 1 hour and 150 mg/kg/hr for 30 minutes until death. At the end of the third hour the blood level was 450 ppm and 6% of the injected subtilin was found in the urine. No symptoms were observed until sometime after the rate was raised to 60 mg, when tremors in the extremities were seen. Death was apparently of cardiac origin. The second animal was given 20 mg/kg/hr for 4 hours. No symptoms were noted, the animal appearing in good condition at the end of this time. The blood level had risen to 700 ppm.

In these 2 rabbits, the blood was observed for agglutination. We had found previously that there was an *in vitro* clumping of red cells at concentrations of subtilin lower than found in these two animals. No clumping was noted, indicating that *in vivo* actions differed from *in vitro*, or that agglutinated cells had been filtered out in the capillary systems of the body.

The third rabbit receiving intravenous infusion was prepared for records of blood pressure and respiration. Approximately 20 mg/kg/hr for 1½ hours did not modify blood pressure, heart rate or respiratory rate. Raising the infusion rate to 70 mg/kg/hr caused a gradual fall in pulse pressure (as measured with a membrane manometer) and a gradual increase in respiratory rate.

Chin<sup>23</sup> has reported that the LD<sub>50</sub> of intravenously injected subtilin to mice is 100 mg/kg. This is slightly higher than the approximately 70 mg/kg found by us in a small series of animals, but either value would

give a body concentration well above the minimum *in vitro* effective tuberculostatic dose. Death from intravenously administered subtilin is presumably due to embolism since subtilin, which dissolves readily in water, is only slightly soluble in physiological saline<sup>18</sup> or serum.<sup>24</sup> The amount held in solution varies somewhat with the manner of manipulation, being around 0.05 to 0.1 g/100 cc under physiological conditions of temperature and salt concentration. The intraperitoneal LD<sub>50</sub> was some 2 to 3 times the intravenous LD<sub>50</sub>. Subcutaneously, mice were able to tolerate more than 3 g/kg of purified subtilin without demonstrable symptoms.<sup>25</sup> The lack of subcutaneous toxicity is likewise explained by the low solubility in body fluids, a deposit of precipitated material being formed at the site of injection.

**Subtilin methyl esters.** The increased solubility of some of the methylated subtilin preparations, and the increased antibiotic activity of others<sup>18</sup> led to the hope that satisfactory blood levels could be obtained. Twelve animals were used. On 4 of them blood assays were not made. There were no untoward reactions in these or any of the other rabbits receiving the esters. It was found that blood levels of the 8 other animals were slightly higher after intravenous, subcutaneous and intramuscular injection of the more soluble ester than the levels found after administration of unmodified subtilin. The levels (up to 2.4 ppm after intramuscular administration) were not great enough to warrant further study. The ester with increased activity was absorbed so poorly that it had an unimportant antibiotic effect in the blood.

Several lots of methyl esters of subtilin were tested for approximate intravenous toxicity in mice. The values obtained did not differ markedly from values for unchanged subtilin.

**Subtilin-pectin complex.** Subtilin, when dissolved with 5 to 8 times its weight of pectin gives a product which is soluble in physiological saline to the extent of 0.5% of subtilin. After some time the complex begins to

<sup>24</sup> Klose, A. A., unpublished data.

<sup>25</sup> Wilson, R. H., Lewis, J. C., and Humphreys, E. M., *Fed. Proc.*, 1948, 7, 266.

break down and subtilin comes out of solution. Dr. Harry S. Owens of this laboratory prepared such a complex for us. It was given intravenously 3 times, intramuscularly and subcutaneously once each. After intravenous injection it disappeared from the blood as rapidly as did unmodified subtilin, and it was as poorly absorbed when given by the other routes. Since it offered no advantages over subtilin and, because of its great viscosity, was very difficult to inject or else required considerable dilution, it was not considered further.

*Pectin-treated subtilin.* At a recent symposium, Salle and Jann<sup>16</sup> stated that a described treatment of subtilin eliminated abscess formation and that subcutaneously injected material so treated was effective against tuberculosis in guinea pigs. The treatment consisted in dissolving 1.5 g of subtilin in 50 ml of 10% urea solution and mixing with an equal volume of 0.1 g of pectin in distilled water. After standing overnight, the supernatant liquid was used for injection. Following this report, we prepared a subtilin solution in the described manner and injected it subcutaneously into 2 rabbits. The dosage was 100 mg/kg, assuming that none of the subtilin was lost during the manipulations. Assays of periodic blood samples showed blood concentrations similar to those reported earlier in this paper.

The question of abscess formation was studied on mice and guinea pigs. We felt that lack of abscesses might be due to simple dilution. Four groups of mice were used, 6 animals to a group. Each mouse received 1 ml of solution under the skin of the back, as follows: (1) 1.5% subtilin, Lot No. 317, in 5% glucose; (2) 5% subtilin No. 317; (3) the solution prepared as described by Salle and Jann, using subtilin No. 317; (4) a 5% solution of subtilin recovered from (3) by precipitation with 10% NaCl.<sup>2</sup> All animals in groups 2 and 4 and a majority in groups 1 and 3 showed abscess formation, the abscesses being smaller and less apt to ulcerate with the more dilute solutions.

Three guinea pigs were used, each animal receiving subcutaneously the following 4 solutions: (1) 1.5 ml of the solution prepared

as described by Salle and Jann; (2) 1.5 ml of 1.5% subtilin No. 317 in 5% glucose; (3) the same *weight* (22 mg) of subtilin in a 10% solution; (4) the same *volume* (1.5 ml) of subtilin in a 10% solution. A large, hard, subcutaneous lump was formed in all 3 animals by solution 4. Solution 3 caused a prompt development of small lumps in 2 animals, and solutions 1 and 2 produced more slowly developing lumps in 1 and 2 animals, respectively. These results indicate that the proposed treatment does not modify the absorption of subtilin nor its tendency to produce abscesses. The decreased reaction was due to the dilution of the material. Since approximately 1/3 of the subtilin was precipitated by the pectin (as estimated by sulphur balance) the concentration of subtilin in the solution prepared according to the method of Salle and Jann was actually about 1%.

*Discussion.* Consideration of the foregoing results indicates that high systemic levels of subtilin are not obtained readily in the rabbit. The amount absorbed after administration in a variety of ways would not produce bactericidal or even bacteristatic levels in the blood stream, except possibly for very sensitive Gram-positive organisms. Furthermore, injection under the skin or into muscle is followed by a lasting deposit which is gradually transformed into an abscess. The poor absorption and the local deposition of precipitated subtilin are caused by the low solubility of subtilin in physiological fluids. It is possible to obtain substantial blood levels in the rabbit only by intravenous administration. If this is done by single injection there is some danger, due again to low solubility causing precipitation in the blood stream, and furthermore, the blood levels are not maintained. The one method of administration which, within the limits of the above experiments, would give a satisfactory blood level with no ill effects to the animal is a slow intravenous infusion.

It is conceivable that results reported here would differ with other species. In the introduction it was noted that certain infections in mice and guinea pigs could be controlled, and we find mice may be killed by intraperitoneal injections of subtilin. However, the

difference is probably only one of degree. In the mouse, as in the rabbit, a subcutaneous deposit is formed, and in the former animal an abscess is formed at the site of injection. Since the lack of absorption and the manifestations of toxicity are dependent on the physiological properties of subtilin in physiological fluids, it seems unlikely that a species difference would be marked.

Such modifications of subtilin as have been tried to date have not greatly modified the physiological absorption or bacteristatic level, even though the products have had somewhat greater *in vitro* antibiotic activity or greatly increased solubility under physiological conditions.

Although the results in this paper indicate that high systemic levels of subtilin are not practical, it is quite possible that subtilin would be beneficial when topical application is indicated. We have observed no reactions which would contraindicate local application.

**Conclusions.** The antibiotic, subtilin, has been administered to rabbits in a variety of ways, with analyses of blood to determine absorption. The bioassay procedures are de-

scribed.

Because of precipitation in physiological fluids, injection into subcutaneous tissue, muscle or peritoneal cavity is an ineffective way of reaching bacteristatic levels of subtilin in the blood stream. Administration by mouth or per rectum is likewise unsatisfactory.

A single injection intravenously is accompanied by some danger and gives a blood level of subtilin which is not maintained. A satisfactory level can be maintained, at least for 4 hours, by slow intravenous infusion, without apparent harm to the animal.

A subtilin-pectin complex, which temporarily allows more subtilin to be soluble in physiological saline, a methyl ester of subtilin which is likewise more soluble and which possesses greater antibiotic activity, and a second methyl ester which is much more soluble, are all absorbed in amounts similar to that of unmodified subtilin.

No observations were made which would contraindicate the topical application of subtilin.

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### 17306. The Antidiuretic Action of Relaxin-Containing Preparations.\*

M. X. ZARROW. (Introduced by Frederick L. Hisaw.)

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The phenomenon of water retention in the female during pregnancy has received a great deal of attention from many investigators. It has been known for some time that both an extract of the posterior pituitary and desoxycorticosterone acetate have antidiuretic action. In addition, Thorn, Nelson, and Thorn<sup>1</sup> have shown that the sex steroids can also produce water retention in the dog.

Nevertheless a suitable explanation for the shift in water balance during pregnancy is still lacking. In the present report data are presented to show that extracts from the ovaries of pregnant sows and blood serum of pregnant rabbits, prepared for relaxative activity on the symphysis pubis of the guinea pig, possess an antidiuretic action in the rabbit.

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<sup>1</sup> Thorn, G. W., Nelson, K. R., and Thorn, D. W., *Endocrinol.*, 1938, 22, 155.

Several different extracts of relaxin were used in these experiments. Preparation J-46 was prepared from unselected ovaries<sup>†</sup> of the sow according to the method of Albert,

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<sup>†</sup> Unselected ovaries were obtained from both pregnant and non-pregnant sows.

Money, and Zarrow<sup>2</sup> and showed an activity of 30 guinea pig units (G.P.U.) per mg dry weight in the symphyseal relaxation test (Abramowitz *et al.*).<sup>3</sup> Two extracts, PS-1<sup>†</sup> and OR-24<sup>§</sup> were prepared from the ovaries of pregnant sows. Pregnant rabbit serum was obtained from rabbits between the 25th and 28th days of pregnancy, and concentrated with alcohol and acetone (Albert and Money).<sup>4</sup> In addition, control extracts were prepared from beef heart and blood serum of male rabbits. Extensive tests on the relaxin preparation J-46 showed that it contained no estrogenic or progestational activity.

All tests for antidiuretic action were carried out in adult female rabbits of the New Zealand strain weighing from 4.0 to 5.5 kg. The rabbits were placed in a metabolism cage and food and water supplied *ad libitum*. In addition the animals received lettuce thrice weekly. Prior to the test, 24 hour values were obtained for the urine output and water intake for about 2 weeks. Only those rabbits were used that showed a fairly constant output of urine during this period. Injections of the test substances were given subcutaneously 3 times a day for 3 days. The antidiuresis occurred usually within 24 hours after the injections were started and definitely by 48 hours and the response was considered positive if the urine output was decreased by 50% or more and maintained at this level for at least the duration of the treatment.

A sample of the data obtained may be seen in Fig. 1 to 4. It will be noted that the injection of either 10 ml of saline thrice daily for 3 days or a beef heart extract had no effect on the urine output. However, the injection of relaxin (J-46) at a dose level of 1000

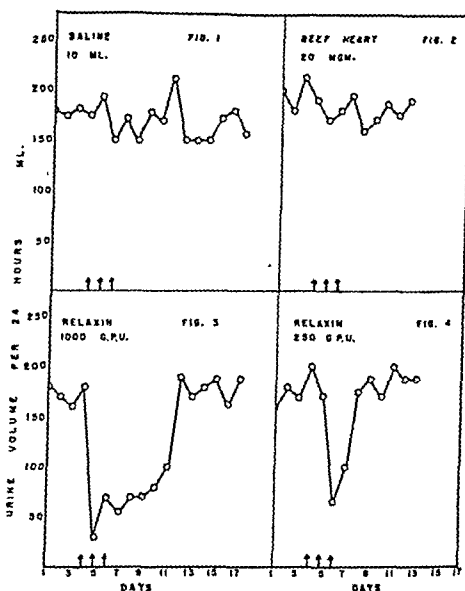


FIG. 1-4.

Twenty-four hour volume of urine in ml plotted against days. Arrows represent days of injection.

Fig. 1. Lack of effect on urine output after treatment with 10 ml of saline injected 3 times daily for 3 days.

Fig. 2. Lack of effect of 20 mg equivalent of beef heart extract injected 3 times daily for 3 days.

Fig. 3. Marked antidiuretic response obtained with 1000 G.P.U. of relaxin (J-46) injected 3 times daily for 3 days.

Fig. 4. Antidiuretic response obtained with 250 G.P.U. of relaxin (J-46) injected 3 times daily for 3 days.

G.P.U. 3 times daily decreased the urine output from 180 ml to approximately 27 ml. The same type of response was also obtained with 250 G.P.U. of relaxin injected in the same manner. However, 100 G.P.U. of relaxin gave no effect.

Thus far we have examined a number of preparations for antidiuretic action and have found that relaxin obtained from the ovaries of pregnant sows, nonselected ovaries<sup>3</sup> and pregnant rabbit serum possesses the ability to cause urine retention (Table I). In addition to the controls mentioned above the injection of blood serum from male rabbits also gave negative results. The minimum effective dose of relaxin for the antidiuretic effect was 250 G.P.U. for our preparations and 50 G.P.U. for the Maltine extract. This discrepancy may be explained by the fact that

<sup>2</sup> Albert, A., Money, W. L., and Zarrow, M. X., *Endocrinol.*, 1947, **40**, 370.

<sup>3</sup> Abramowitz, A. A., Money, W. L., Zarrow, M. X., Talmage, R. V. N., Kleinholz, L. H., and Hisaw, F. L., *Endocrinol.*, 1944, **34**, 103.

<sup>†</sup> Obtained through the courtesy of Dr. Edward H. Frieden, Biological Laboratories, Harvard University.

<sup>§</sup> Obtained through the courtesy of Dr. Robert L. Kroc, Maltine Company, Morris Plains, N. J.

<sup>4</sup> Albert, A., and Money, W. L., *Endocrinol.*, 1946, **38**, 56.

TABLE I.  
Antidiuretic Effect of Relaxin Preparations in the Rabbit.

Relaxin	Dose in G.P.U.*	Source of relaxin	No. of tests	Response
J-46	3000	Ovaries of sows	3	Positive
"	1000	" " "	4	"
"	500	" " "	4	"
"	250	" " "	4	"
"	100	" " "	2	Negative
PS-1	500	Ovaries of pregnant sows	1	Positive
OR-24	1000	Ovaries of pregnant sows	1	Positive
"	500	" " " "	1	"
"	250	" " " "	1	"
"	100	" " " "	1	"
"	50	" " " "	2	"
"	25	" " " "	1	Negative
PR-1	500	Blood of pregnant rabbits	1	Positive
Control	10 ml	Saline	3	Negative
"	1 ml	"	1	"
"	2 ml	"	2	"
"	20 mg	Beef heart	2	"
"	50 ml †	Blood of male rabbits	2	"

\* Represents the individual dose that was injected 3 times daily for 3 days.

† The equivalent of 50 ml of blood was injected 3 times daily.

the Maltine unit appears to be approximately 4 times greater than our unit. Thus it would seem that the antidiuretic activity parallels the relaxin activity in extracts prepared in two different laboratories. Furthermore, the presence of the antidiuretic activity in relaxin preparations obtained from such diverse sources as the ovary of the sow and the blood of pregnant rabbits appears to be highly significant. While the evidence is as yet insufficient, there is this striking correlation between the content of relaxin in the preparation and the ability to induce water retention. It may also be pointed out that this new antidiuretic factor is not identical with the posterior pituitary hormone. Donaldson<sup>5</sup> has shown that the latter is dialyzable whereas relaxin and the antidiuretic factor are non-dialyzable.<sup>3</sup> The probability that relaxin is responsible for water retention is also supported by the fact that the blood serum of pregnant rabbits produces both relaxation of the symphysis pubis and water retention

whereas the blood serum of male rabbits is without these effects.

The possible identity of the antidiuretic action with relaxin and the fact that the latter substance is found primarily in high concentrations during pregnancy,<sup>6-8</sup> leads to speculation as to whether relaxin may be concerned with the shift in water balance during gestation.

**Summary.** Relaxin containing extracts of the ovaries of pregnant sows and of the blood of pregnant rabbits possess an antidiuretic action in the rabbit. Some evidence is presented to indicate that the antidiuretic activity parallels the relaxin activity of the preparations used in the present study.

<sup>6</sup> Marder, S. N., and Money, W. L., *Endocrinol.*, 1944, **34**, 115.

<sup>7</sup> Zarrow, M. X., *Proc. Soc. Exp. Biol. and Med.*, 1947, **66**, 488.

<sup>8</sup> Hisaw, F. L., and Zarrow, M. X., *Proc. Soc. Exp. Biol. and Med.*, 1948, **69**, 395.

Received July 5, 1949. P.S.E.B.M., 1949, **71**.

<sup>5</sup> Donaldson, W., *J. Clin. Invest.*, 1947, **26**, 1023.

# 17307. A Chemical Method for the Detection of Virus Infection of the Chick Embryo.

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Chemical or physical differences in the allantoic fluid of normal and virus-infected chick embryos have been emphasized by few investigators. McLean *et al.*<sup>1</sup> mentioned differences in the pH of normal and influenza virus-infected allantoic fluids. Parodi and his collaborators<sup>2</sup> reported a slower decline in pH and an increase in volume of the allantoic fluid from embryos infected with influenza virus. In studies with their common cold virus (MR-1) Atlas and Hottle<sup>3</sup> noted high absorption peaks with dialyzed infected allantoic fluid which were attributed to protein. Such peaks were sometimes observed with normal fluid. In view of such observations, it appeared possible that there might be sufficient chemical difference in virus-infected and normal allantoic fluid to permit the development of a chemical test for virus infection of the chick embryo.

Early in the course of a systematic investigation of the properties of infected and normal allantoic fluid, it was discovered that allantoic fluid from embryos infected with influenza virus contained appreciably greater quantities of protein. Accordingly, a simple quantitative method for the determination of protein in allantoic fluid was devised and subsequently utilized in studies of infection of the allantoic sac with various viruses.

**Materials and methods.** *Allantoic fluid.* Allantoic fluids used in turbidity determinations were carefully harvested from 10-12-day-old White Leghorn embryos previously chilled for 12-18 hours at 4°C. Grossly bloody fluids and those inadvertently contaminated by yolk were discarded. Groups of 5-6 embryos were employed.

*Viruses.* The PR8 and Lee strains of influenza virus and a strain of Newcastle disease virus adapted to the allantoic sac by serial passage were used. The Habel strain of mumps virus, adapted in this laboratory to the allantoic sac, was also utilized. Semliki Forest virus (SFV) in the form of desiccated mouse brain (110th passage) was obtained through the courtesy of Dr. K. C. Smithburn, who had demonstrated<sup>4</sup> rapid multiplication of the virus in the chick embryo. This mouse brain suspension killed embryos within 24-36 hours when injected into the allantoic sac. Subsequent passages were made with allantoic fluid.

*Control materials.* Allantoic fluids, hereinafter referred to as normal, were obtained from embryos inoculated with normal or heated (65°C for 30 minutes) allantoic fluid diluted 1:10 to 1:1,000 in 0.85% sodium chloride solution buffered to pH 7.2 with phosphate. The same diluent was used for virus inocula.

*Turbidity determination.* One cc of 10% trichloroacetic acid is added to 1 cc of allantoic fluid in a soft glass test tube measuring 100 × 10 mm. Reagents are measured with ordinary serologic pipettes. Two to 5 minutes after the addition of acid, turbidity readings are determined in a Klett-Summerson colorimeter. An adapter for the small test tube and a blue filter (peak transmittance, 420 mμ) are employed. Mixing of reagents is accomplished by inversion of the colorimeter tube. A blank of 10% trichloroacetic acid is used for preliminary setting of the zero reading of the colorimeter. In the instrument used in this study a 0.03% suspension of barium sulfate gives a reading of 70. Turbidity is expressed directly in the units comprising the scale of the colorimeter, these units being directly proportional to optical density. Fifteen serial determinations of

<sup>1</sup> McLean, I. W., Jr., Cooper, G. K., Taylor, A. R., Beard, D., and Beard, J. W., *Proc. Soc. Exp. Biol. and Med.*, 1945, **59**, 192.

<sup>2</sup> Parodi, A. S., Lajmanovich, S., Pennimpede, F., and Mittelman, N., *J. Immunol.*, 1948, **58**, 109.

<sup>3</sup> Atlas, L., and Hottle, G., *Science*, 1948, **108**, 743.

<sup>4</sup> Smithburn, K. C., *J. Immunol.*, 1946, **52**, 309.

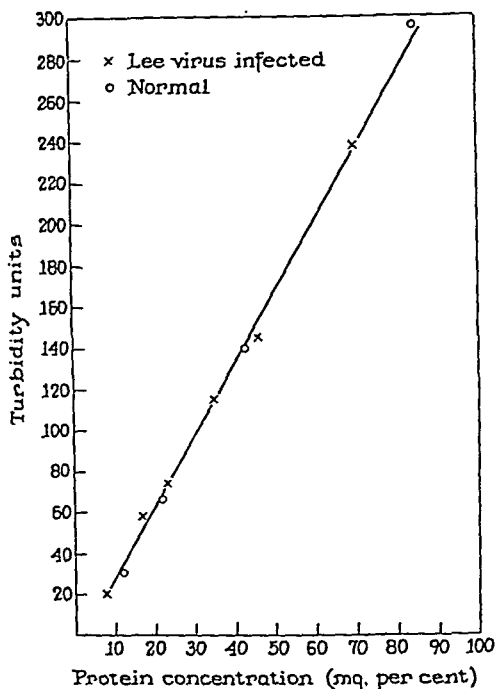


FIG. 1.

Relation between turbidity and protein concentration in allantoic fluid. Turbidity was produced with 10% trichloroacetic acid. Protein  $N_2$  was determined by micro-Kjeldahl.

turbidity, using the same allantoic fluid pool, showed an experimental error of  $\pm 2.8\%$  for the procedure as described above.

**Experimental.** Studies of dialyzed allantoic fluids revealed a substance precipitable by 10% trichloroacetic acid to be present in both normal and influenza virus-infected fluids and greatly increased in infected fluids. Similarly, micro-Kjeldahl determinations of total nitrogen demonstrated a higher concentration of nitrogen in fluid from infected embryos. The linear relation of the turbidity with trichloroacetic acid and protein concentration of both normal and infected allantoic fluids may be seen in Fig. 1 in which the turbidities of varying dilutions of concentrated dialyzed allantoic fluids are plotted against the protein concentrations of the fluids as determined by the micro-Kjeldahl method. This relationship was found to hold with concentrations up to 500 turbidity units. However, specimens giving readings above 300 were diluted and re-examined because of

difficulties experienced in obtaining accurate scale readings in the higher range. A similar straight line relationship between serum protein concentration and turbidity produced by trichloroacetic acid has been established within certain limits of concentration by Chow *et al.*<sup>5</sup>

Further study of the acid-precipitable substance in allantoic fluid has indicated its protein nature. Dialysis in cellophane against 0.85% saline did not reduce the concentration of the substance. The characteristic biuret, xanthoproteic, and ninhydrin color reactions were given by dialyzed allantoic fluids. Precipitates were formed in dialyzed and non-dialyzed fluids by the addition of 95% ethyl alcohol or ammonium sulfate. The antigenicity of the substance, which is discussed below, is further evidence for its protein nature.

Fractionation of dialyzed allantoic fluids by half and full saturation with ammonium sulfate was performed. The protein concentration of these fractions was then determined by micro-Kjeldahl analysis after re-dialysis against saline. The albumin-globulin ratios in normal and infected fluids did not

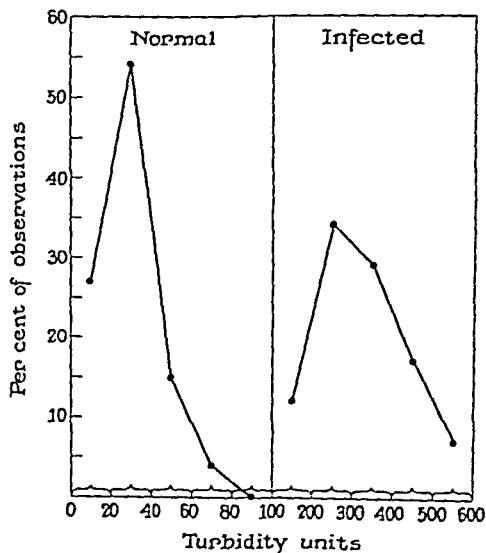


FIG. 2.

Frequency distribution of turbidity values with normal and Lee virus infected allantoic fluids.

<sup>5</sup> Chow, B. F., Hall, L., Duffy, B. J., and Alper, C., *J. Lab. and Clin. Med.*, 1948, **33**, 1440.



TABLE I.  
Increase in Allantoic Fluid Protein with Various Viruses.

Virus 10-3 dilution inoculated	Time after inoculation, days	No. of eggs	Allantoic fluid turbidity†	
			Range	Mean
Lee	2	41	118-540	316
SFV*	1-1½	17	40-300	119
NDV	2	20	52-260	104
PR8	2	12	30-152	87
MV	4-5	12	56-160	84
Control	2	100	8-75	29
"	4-5	10	21-50	33

\* 10-1 to 10-3 dilutions used.

† Turbidity developed with 10% trichloroacetic acid.

differ materially, being 7.3/1 in normal and 8/1 in infected fluids. The addition of trichloroacetic acid to fractions obtained by ammonium sulfate precipitation produced turbidity equivalent to the protein nitrogen concentration. Ultraviolet absorption curves obtained with the Beckman spectrophotometer disclosed minima of 252 and 290  $m\mu$  and maxima of 265  $m\mu$  with both dialyzed normal and infected fluids of equal protein concentration (40 mg %). Absorption in this range is characteristic of proteins.

Electrophoretic studies\* were made of dialyzed normal and infected allantoic fluids. Normal fluid was concentrated 23-fold, and infected fluid 9-fold, resulting in protein concentrations of 375 and 300 mg %, respectively. These concentrations proved insufficient for accurate analysis of mobilities or sharp delineations of peaks; however, the rate of boundary migration and the degree of boundary spreading were consistent with what would be expected with protein solutions containing several components. With both normal and infected fluids at least 3 peaks were clearly discernible.

*Comparison of normal and infected allantoic fluid protein concentrations.* Studies of uninfected embryos demonstrated low concentrations of protein in the allantoic fluid as measured by turbidity produced with trichloroacetic acid. In embryos of 10-12 days of age turbidity values varied from 8 to 75,

representing protein concentrations of 2.4 to 23 mg % with an average value of 8.7 mg %. The frequency distribution of turbidity readings of allantoic fluids from 100 normal embryos is charted in Fig. 2 and compared with the range of turbidity (*i.e.* protein concentration) found in fluids from 41 embryos infected with the Lee strain of influenza virus. Turbidity readings of the infected allantoic fluids ranged from 118 to 540 (*c.f.* Table I), indicating concentrations of 35.4 to 162 mg % of protein.

Lee-infected allantoic fluid was subjected to differential centrifugation to determine to what extent sedimentable substances contributed to turbidity produced with trichloroacetic acid. Low speed centrifugation (3,000 r.p.m. for 10 minutes) caused a 12% reduction in the turbidity observed initially, suggesting the presence of considerable cellular material. Total cell counts disclosed an average of 560 cells/cu mm in Lee-infected allantoic fluid contrasted with an average of 93 cells/cu mm in normal fluid. Further centrifugation at 37,900 g for 30 minutes resulted in a further reduction of the turbidity produced with acid amounting to 8% of the turbidity originally present. Thus, Lee virus, itself, contributes little, if any, to the turbidity of infected allantoic fluid, as this amount of centrifugation leaves less than 1% of the virus in the supernate.

*Source of protein in infected allantoic fluid.* It appeared likely that the increased protein in the allantoic fluid of virus-infected embryos was attributable to host reaction and perhaps to destruction of host tissue, especially in

\* Electrophoretic analysis was kindly carried out by Dr. Gertrude E. Perlmann, The Rockefeller Institute, New York City.

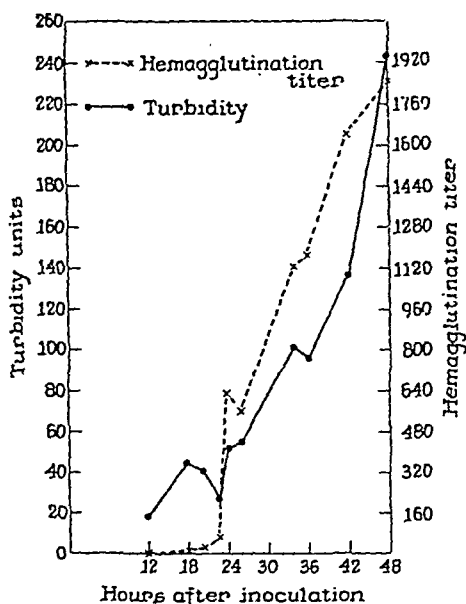


FIG. 3.

Temporal relation between increase in turbidity and Lee virus concentration in allantoic fluid.

view of the increased number of cells in infected fluid. Evidence cited above demonstrated that the virus itself did not contribute to the turbidity produced with acid, and further studies have shown no direct relation between virus and protein concentrations. Moreover, experiments with Lee virus demonstrated a difference in the rate of virus multiplication and the increase in allantoic fluid turbidity as is shown graphically in Fig. 3. Corroboration of this difference was obtained in experiments in which the time required to reach maximal virus concentration was varied by the use of inocula of differing dilutions of virus. The results are presented in Table II.

The protein of infected allantoic fluid did not differ immunologically from the protein normally present in allantoic fluid. Sera from rabbits injected intravenously with dialyzed infected or normal allantoic fluid contained antibodies capable of forming precipitates with concentrated allantoic fluid from either infected or normal embryos. These antibodies were absorbed from either antiserum by normal or infected allantoic fluid antigen, as well as by a suspension of normal chorio-allantoic membrane (C.A.M.). These data are summarized in Table III.

*Viruses which cause an increase in allantoic fluid protein.* The Lee and PR8 strains of influenza virus, Semliki Forest virus, Newcastle disease virus, and mumps virus consistently caused an increase in allantoic fluid protein during the course of infection of the allantoic sac. A correlation may be drawn between the toxicity of the viruses studied and the degree of protein increase. Lee and Newcastle disease viruses, which killed embryos after 48 hours, and Semliki Forest virus, which killed even sooner, caused significantly more turbidity in allantoic fluid than did the considerably less toxic PR8 and mumps strains, as is shown in Table I.

*Nonviral causes of protein increase in allantoic fluid.* The production of the turbidity reaction by 5 different viruses is evidence of its non-specificity. It is obvious that any reaction dependent upon response or destruction of host tissue may be induced by chemical or physical agents as well as infectious ones. Thus, it was found that injection of 0.1 cc quantities of broth or serum might increase the allantoic fluid protein of embryos beyond the low concentrations usually seen. Such increases were greater than would be anticipated on the basis of the amount of protein injected, demonstrating an actual reaction of the embryonic tissue to the material introduced.

When broth was injected in control embryos, mean turbidity values were almost double (*i.e.* 48 units) those observed in saline inoculated embryos, and occasional fluids exceeded 100 turbidity units. The injection of undiluted allantoic fluid occasioned similar non-specific response, although it was of lesser degree (mean turbidity, 38 units), and only 10% of individual fluids exceeded the upper limit of 75 turbidity units observed in normal embryos (Table I).

The effect of bacterial infection of the chick embryo was not systematically studied, but examination of bacterially contaminated allantoic fluids disclosed increased turbidity, *i.e.* more than 75 units, in only 2 of 16 instances. In any event, such fluids are unsuitable for virus study, and are customarily discarded.

In normal embryos incubated for more

TABLE II.  
Relation of Increase in Allantoic Fluid Turbidity to Extent of Multiplication of Lee Virus.

Virus dilution inoculated	Time after inoculation					
	24 hr		36 hr		48 hr	
	Hem. titer*	Turb.†	Hem. titer	Turb.	Hem. titer	Turb.
10-3	1:636	53	1:1331	96	1:1843	243
10-5	0	28	1:1536	93	1:2048	218
10-7	0	21	1:65	42	1:1229	145

\* Mean hemagglutination titer of allantoic fluids.

† Mean turbidity value of allantoic fluids.

TABLE III.  
Absorption Experiments with Antisera Against Normal and Infected Allantoic Fluid Protein.

Rabbit serum		Precipitin titer*	
Absorbed with		Normal all. fl. prot.	Infected all. fl. prot.
Anti normal all. fl. prot.	—	1:250	1:250
" " " " " "	Infected all. fl. prot.	0	0
Anti infected all. fl. prot.	—	1:250	1:250
" " " " " "	Normal all. fl. prot.	0	0
" " " " " "	Normal C.A.M.	0	0

\* Highest dilution of antigen which gave a positive reaction with serum diluted 1:2.

TABLE IV.  
Prevention of Protein Increase in Allantoic Fluid by Virus Antiserum.

Inoculum		Mean hemagglutination titer of allantoic fluids	Mean turbidity value of allantoic fluids
Serum	Lee virus dilution		
—	10-6	1:1877	150
Normal 1:100	"	1:2046	93
" 1:500	"	1:2253	188
Anti Lee 1:100	"	0	25
" " 1:500	"	1:2	19
" " 1:100	—	—	20
" " 1:500	—	—	42

than 12 days, sharp, capricious increases in the allantoic fluid protein may occur, making such embryos unsuitable for use with the present method. Fluids grossly contaminated by blood introduced at the time of harvest also proved useless because of the presence of extraneous serum protein.

*Prevention of protein increase by virus antiserum.* Neutralization of Lee virus by specific immune rabbit serum prevented the increase in allantoic fluid protein which occurred following the injection of virus and normal serum or virus alone. The results are

summarized in Table IV. It will be seen that, in the dilutions indicated, inactivated rabbit serum *per se* caused no undue elevation of mean turbidity. In other experiments dilutions of rabbit serum as low as 1:40 were used without increase in allantoic fluid protein. This experiment affords definitive evidence that the protein increase which follows introduction of virus into the allantoic sac is a corollary of virus multiplication.

*Discussion.* The study of many animal viruses and attempts to recover new viruses have been handicapped by the lack of simple

*in vitro* technics comparable to the hemagglutination reaction. The method outlined in the present communication has the virtue of simplicity, and in theory may be of value in the detection of any virus capable of multiplication in the cells of the allantoic sac of the chick embryo. Because the method is dependent upon the vagaries of host response, it is unsuitable for the direct measurement of virus concentration. However, both specificity and quantitation may be obtained by the employment of immune serum. It is conceivable that a virus might be recovered and identified immunologically as the etiological agent of a disease solely by chemical evidence of infection of the chick embryo.

**Summary.** A method is described for the detection of virus infection of the allantoic sac of the chick embryo. The method is dependent upon the increased concentration of protein in infected allantoic fluid. Protein concentration is measured by determining the degree of turbidity produced upon the addition of 10% trichloroacetic acid to allantoic fluid.

While this article was in press, Polson and Dent (*Nature*, 1949, **164**, 233) described increases in the protein concentration of allantoic fluid from eggs infected with lumpy skin disease virus or blue tongue virus.

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### 17308. Hemagglutination with the GDVII Strain of Mouse Encephalomyelitis Virus.

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The capacity of certain viruses to cause agglutination of erythrocytes<sup>1</sup> has permitted the development of *in vitro* procedures which have greatly facilitated investigative and diagnostic work with these agents. Although at least 10 different animal viruses are known to cause hemagglutination (pertinent data have been summarized recently),<sup>2</sup> there is almost no evidence indicating that any of the neurotropic viruses possesses a similar capacity. Recently, however, Bremer and Mutsaers<sup>3</sup> stated that the Lansing strain of poliomyelitis virus caused agglutination of sheep RBC, and Hallauer<sup>4</sup> stated that Columbia SK and Columbia MM viruses also agglutinated sheep RBC. We have been unable to confirm

the results reported for the Lansing strain, as too have other workers.<sup>5</sup> However, in the accompanying paper Olitsky and Yager<sup>5</sup> have confirmed and extended the results reported for SK and MM viruses.

The present study was concerned chiefly with the GDVII strain of mouse encephalomyelitis virus as well as with the FA strain.<sup>6</sup> In addition, the Lansing, MEF1, and Brunhilde strains of poliomyelitis virus were investigated. It will be demonstrated that the GDVII strain causes agglutination of human RBC at 4°C, but not at 23 or 37°C; that such hemagglutination is inhibited by homologous immune serum, and by anti-FA virus serum, but not by antiserum against other viruses. No evidence of hemagglutination could be obtained with the FA strain nor with any of the strains of poliomyelitis virus which were employed.

#### *Materials and methods. Viruses. The*

\* Aided by a Fellowship from the National Foundation for Infantile Paralysis.

<sup>1</sup> Hirst, G. K., *J. Exp. Med.*, 1942, **75**, 49.

<sup>2</sup> Smadel, J. E., *Viral and Rickettsial Infections of Man*, 1948, chap. 3, J. B. Lippincott Co., Philadelphia, Pa.

<sup>3</sup> Bremer, A., and Mutsaers, W., *Compt. rend. Soc. Biol.*, 1948, **142**, 1192.

<sup>4</sup> Hallauer, C., 4th Internat. Cong. Microbiol., July 20-26, 1947, Copenhagen, 1949, p. 257.

<sup>5</sup> Olitsky, P. K., and Yager, R. H., accompanying paper.

<sup>6</sup> Theiler, M., and Gard, S., *J. Exp. Med.*, 1940, **72**, 49.

GDVII strain was obtained from Dr. Max Theiler, I.H.D. Laboratories, The Rockefeller Foundation, New York City. Three FA strains were used; one obtained from Dr. Theiler and 2 obtained from Dr. J. Melnick, Yale University, New Haven, Conn. Three poliomyelitis virus strains were employed; the Brunhilde strain was kindly supplied by Dr. D. Bodian, The Johns Hopkins University, Baltimore, Md., and the Lansing and MEF1 strains were obtained from Dr. P. K. Olitsky, The Rockefeller Institute, New York City. The Brunhilde strain was contained in infected monkey spinal cord. The other viruses were maintained by occasional intracerebral passage in mice. Brains were removed from exsanguinated mice shortly after the appearance of signs indicating infection of the central nervous system. Ten per cent brain suspensions were prepared with 0.01 M phosphate buffer at pH 7.2. The suspensions were ground for 2½ minutes in a modified Waring Blendor which was cooled with ice and then were centrifuged for 15 minutes at 7,760 g. The supernates were employed either promptly after preparation or following storage at -70°C, sometimes for as long as 14 days. Virus titrations were performed by the intracerebral technic using serial 10-fold dilutions in 10% normal rabbit serum saline. A group of 5 or 6 mice was used for each dilution and the 50% infectivity end point, LD<sub>50</sub>, was calculated in the usual manner.

**Hemagglutination technic.** Hemagglutination titrations were carried out in a manner similar to that employed with influenza virus.<sup>1</sup> Serial 2-fold dilutions of brain suspensions in saline buffered at pH 7.2 and a final concentration of 0.25% human Group O erythrocytes were employed. With the GDVII strain the mixtures were held at 4°C for 2 hours. Readings were recorded in the usual manner and the end point was taken as the highest dilution which gave a 2+ reaction.

**Hemagglutination-inhibition technic.** Antibody titrations were carried out with serial 2-fold dilutions of inactivated (56°C/30 min.) sera in buffered saline and a constant amount of virus, usually 16 hemagglutinating units. A final concentration of 0.25% RBC

TABLE I.  
Hemagglutination with GDVII Strain of Mouse Encephalomyelitis Virus.

Supernate of mouse brain suspension	Human group O erythrocytes, %	Held 2 hr at °C	Final dilution of supernate									
			135	250	500	1000	2000	4000	8000	16,000	32,000	64,000
GDVII strain	0.25	4	4*	4	4	4	4	4	3	1	±	0
" "	0.25	23	0	0	0	0	0	0	0	0	0	0
" "	0.25	37	0	0	0	0	0	0	0	0	0	0
" "	2.5	4	4	4	2	0	0	0	0	0	0	0
" "	1.0	4	4	4	4	3	2	1	1	±	±	0
" "	0.5	4	4	4	4	4	4	4	3	1	±	0
FA "	0.25	4	4	4	4	4	3	2	1	0	0	0
Poliomyelitis, Lansing	0.25	4	4	4	4	4	4	4	4	4	4	0
" MEF1	0.25	4	0	0	0	0	0	0	0	0	0	0
Normal, control	0.25	4	0	0	0	0	0	0	0	0	0	0
" "	0.25	4	0	0	0	0	0	0	0	0	0	0

\* Indicates degree of hemagglutination.

was used and readings were made after 2 hours at 4°C. The end point was taken as the highest dilution of serum which completely inhibited hemagglutination.

*Immune sera.* Through the courtesy of Dr. P. K. Olitsky immune sera against a large number of different neurotropic viruses were made available. In most instances the sera were obtained from rabbits which had been repeatedly injected intraperitoneally or subcutaneously with infected mouse brain. In some instances sera were also obtained from immunized guinea pigs, mice or monkeys. Immune sera and control normal sera usually were stored at -30°C.

*Hemagglutination with GDVII virus.* Positive results were obtained in hemagglutination experiments with the GDVII strain when (a) suspensions of infected mouse brain, (b) human Group O erythrocytes, and (c) a reaction temperature of 4°C were employed. The results of typical experiments are shown in Table I. High titers ranging from 1:2,000 to 1:16,000 or more were commonly obtained with 0.25% RBC. In general, the hemagglutination titer was inversely proportional to the concentration of RBC, as is the case also with influenza virus.<sup>7</sup> Hemagglutination occurred only if the mixtures were cold (4°C), disappeared rapidly when cold mixtures were warmed either at room temperature (23°C) or at 37°C, and reappeared when the mixtures were again cooled to 4°C. The reaction developed relatively slowly and, although clear evidence of hemagglutination was present at one hour, more definite agglutination was present at 2 hours. The pattern of agglutinated cells was closely similar to that observed with human RBC and either influenza or mumps virus.

Despite numerous attempts employing a wide range of experimental conditions, it was not possible to obtain evidence of hemagglutination with the FA strain nor with the Lansing, MEF1 or Brunhilde strains of poliomyelitis virus. In addition to human RBC, erythrocytes from the following species were used: monkey, horse, sheep, cat, dog, guinea pig, hamster, mouse and chicken. The GDVII strain was incapable of causing ag-

glutination of any RBC other than those derived from man. Supernates of normal mouse brain suspensions, prepared as described above, did not cause agglutination of human RBC at dilutions greater than 1:4. As is pointed out also in the accompanying paper,<sup>6</sup> erythrocytes of certain species, *e.g.*, hamster, dog, cat and guinea pig, as well as the mouse, commonly showed agglutination when mixed with normal mouse brain suspensions.

The agglutination of human RBC which is caused by GDVII virus in the cold disappears after a few minutes at room temperature. Because of this, both the reaction and readings of titrations are best carried out in the cold room. As is shown below, agglutination is associated with adsorption of the virus to RBC and the dispersal of the agglutinated cells is associated with elution of the virus from them. Successive cycles of adsorption and elution, dependent merely on changes in temperature, can be repeated at will with a single mixture of RBC and GDVII virus. Four such cycles have been carried out.

*Hemagglutination-inhibition with immune serum.* Agglutination of human RBC with GDVII virus in the cold was prevented by high dilutions of anti-GDVII serum as well as anti-FA serum but not by immune serum against other viruses. The results of typical experiments are shown in Table II. Hemagglutination-inhibition titers ranging from 1:4,000 to 1:16,000 were obtained commonly with anti-GDVII serum and similar high titers were obtained also with anti-FA serum. Normal serum usually caused some non-specific inhibition; with rabbit and guinea pig serum titers of 1:32 or lower were commonly found; with human and mouse serum titers as high as 1:128 were encountered. In some instances heating at 56°C for 30 minutes reduced the degree of non-specific inhibition. It should be emphasized that the injection of mouse brain suspensions into animals other than mice commonly results in the development of antibodies which cause agglutination of human RBC. Usually the agglutination titer of such sera is not greater than 1:128 but in occasional instances it may be considerably higher. With immune serum agglutination of RBC occurs not only at 4°C but also

<sup>7</sup> Whitman, L., *J. Immunol.*, 1947, 56, 167.

TABLE II.  
Inhibition of Hemagglutination with GDVII Virus by Immune Serum.

Immune vs.	Serum	Species	GDVII virus units	Held 2 hr at °C	Serum hemagglut. inhibition titer
Normal m.br.*		Rabbit	16	4	0†
GDVII m.br.		"	16	4	8000
Normal m.br.		Mouse	16	4	0
FA m.br.		"	16	4	8000
" "		"	16	4	8000
Poliomyelitis (conv.)		Monkey	16	4	0
" "		"	16	4	0
" "	Lansing m.br.	Rabbit	16	4	0
Mengo, m.br.		"	16	4	0

\* m.br. = Mouse brain.

† 0 = No inhibition of hemagglutination at 1:32 serum dilution.

at room temperature or at 37°C, as not with GDVII virus. The agglutinins were readily removed from such sera by absorption with 20% human RBC at 4°C. Adsorbed immune sera gave hemagglutination-inhibition titers with GDVII virus which were identical with those obtained with unadsorbed sera.

Immune sera against the following viruses were employed in hemagglutination-inhibition experiments with GDVII virus: lymphocytic choriomeningitis, Eastern equine encephalitis, Japanese B encephalitis, St. Louis encephalitis, Russian Far East encephalitis, vesicular stomatitis, West Nile, rabies, herpes simplex, vaccinia, Columbia SK, Columbia MM, Men-go encephalomyelitis, encephalomyocarditis, influenza A (PR8 strain) and PVM. In no instance was significant inhibition of GDVII virus demonstrable with these sera.

In cross-immunity experiments Theiler and Gard<sup>6</sup> demonstrated an immunological relationship between GDVII and FA viruses. It appears of considerable interest that the results of hemagglutination-inhibition experiments indicate not only that antibody specifically directed against GDVII virus is present in high titer in immune sera but also show clear evidence of a close antigenic relationship to FA virus.

**Adsorption and elution of GDVII virus.** When mixtures of human RBC and GDVII virus were held at 4°C, the virus was adsorbed rapidly by the erythrocytes and sedimented with them on light centrifugation. When the sedimented RBC were resuspended in buffered saline and held at 4°C, elution of the virus did not occur. However, when the re-

suspended RBC were warmed to 37°C, elution of the virus occurred very rapidly and maximum titers were obtained in the supernate within 5 to 10 minutes. Results of typical experiments are shown in Fig. 1. As would be expected, the concentrations of RBC employed affected the extent to which the virus was adsorbed at 4°C but did not have any striking effect on the rate or degree of elution at 37°C. Mixtures held at 4°C for as long as 24 hours showed no significant elution of the virus from RBC.

That hemagglutination with GDVII virus is caused by the virus particle itself and not by a component separable from the virus is

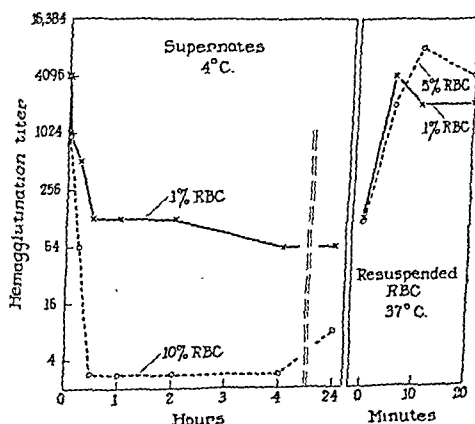


FIG. 1.

Adsorption of GDVII virus on human Group O RBC at 4°C and elution at 37°C. Hemagglutination titer of supernates from mixtures of virus and RBC is plotted against time mixtures were held at 4°C. Hemagglutination titer of supernates from resuspended RBC is plotted against time such erythrocytes were held at 37°C.

TABLE III  
Adsorption on and Elution from Human RBC of GDVII Virus.

Material tested	Hemagglutination titer* at 4°C vs. human RBC	Virus infectivity titer* in mice I.C., LD <sub>50</sub>
GDVII m.br. suspension	10,240	10-8.1
Supernate after adsorption with 10% RBC, 1 hr at 4°C	160	10-5.6
Supernate of resuspended RBC in saline, 15 min. at 37°C	10,240	10-7.8

\* Titers are expressed in terms of final dilution of brain material.

indicated by the results shown in Table III.

Adsorption of a suspension with human RBC at 4°C resulted in reductions in the hemagglutination and virus infectivity titers of the supernate which were of similar degree. Moreover, on warming the resuspended RBC at 37°C, similar increases in both titers occurred indicating that elution of the virus was effected at the higher temperature.

*Properties of hemagglutination component.* The hemagglutination titer of GDVII mouse brain suspension was not diminished by storage at 4°C for 43 days. Heating crude suspensions in saline at 56°C for 30 minutes caused marked loss, i.e. 99%, of hemagglutinating capacity. On the other hand, suspensions prepared from brain material extracted by methanol in the cold showed only a 2- to 4-fold reduction in titer on similar heating. Moreover, such suspensions showed hemagglutination titers of 1:1,000 after heating at 65°C for 30 minutes. Centrifugation at 7,760 g for 30 minutes did not reduce the hemagglutination titer of suspensions. Filtration through Seitz-EK pads caused an 8-fold reduction in the titer of the filtrate. Suspensions buffered at pH values from 4.8 to 8.3 gave similar titers. The amount of virus adsorbed by human RBC at 4°C was not significantly affected by the pH of the mixture within this range. Furthermore, elution of virus from RBC at 37°C was complete when erythrocytes were resuspended in buffer of pH 4.8 to 8.

*Concentration of GDVII virus.* By resuspension of RBC with adsorbed virus in small volumes of buffered saline and warming the suspension to 37°C, it was possible to achieve considerable concentration (10 times or

more) of the virus in the eluate. In most instances the increase in titer obtained was as great as or greater than would have been expected in terms of the volumes of eluate employed. Either high or low titer suspensions as well as suspensions which had been diluted before adsorption yielded satisfactory results in concentration experiments of this kind.

Recently it was reported<sup>8</sup> that GDVII virus could be purified considerably by precipitation with 25 to 30% methanol in the cold. In the present study numerous attempts were made to concentrate the virus by means of such a procedure. In all instances the virus titer was determined by the hemagglutination technic. It was found that 52% methanol mixtures held at 4°C for 3 hours yielded better results than mixtures at other methanol concentrations. Despite the use of a large variety of experimental conditions which included variations in pH, ionic strength, amount of centrifugation before and after the addition of methanol, as well as extraction of brain material with organic solvents, it was not possible to obtain consistent results. In some experiments 10-fold or greater concentration was achieved but the results were not sufficiently reproducible to make the procedure valuable. Moreover, after concentration by methanol precipitation, the virus appeared to be unstable and hemagglutination titers decreased rapidly on storage of concentrated material at 4°C.

*Failure of FA virus to cause hemagglutination.* The infectivity titer of GDVII virus is

<sup>8</sup> Brumfield, H. P., Stulberg, C. S., and Halvorson, H. O., *Proc. Soc. Exp. Biol. and Med.*, 1948, 68, 410.



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" "	Monkey	16	4	8000
Polio myelitis (conval.)	"	16	4	0
" " Lansing m.br.	Rabbit	16	4	0
Mengo, m.br.	"	16	4	0

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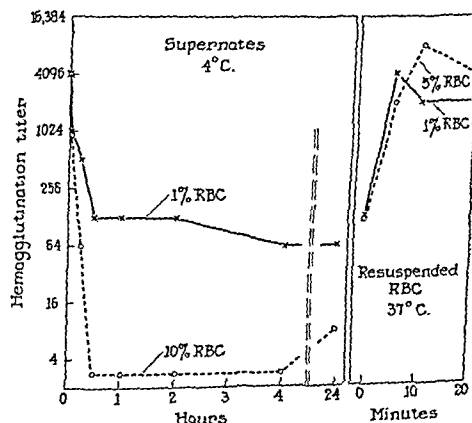


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# 17309. Hemagglutination by Columbia SK, Columbia MM, Mengo Encephalomyelitis and Encephalomyocarditis Viruses: Experiments with Other Viruses.

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During the course of a study on hemagglutination by certain neurotropic viruses, a study suggested by the findings reported in the foregoing paper,<sup>1</sup> the writers' attention was directed to two reports. One<sup>2</sup> stated that the Lansing strain of poliomyelitis virus, and the other<sup>3</sup> that the Columbia SK (Col SK) and Columbia MM (Col MM) viruses agglutinated sheep erythrocytes, the agglutination being inhibited by specific antisera.

In our own work no hemagglutination by the Lansing strain was found; Hallauer<sup>3</sup> also reported failure. On the other hand, hemagglutination of sheep RBC by Col SK and Col MM viruses<sup>3</sup> was not only confirmed in the present investigation but a similar specific reaction was also obtained with Mengo encephalomyelitis (ME) and encephalomyocarditis (EMC) viruses.

This paper reports the results of these tests as well as attempts to disclose agglutination of sheep red cells and several additional types of erythrocytes by still other neurotropic viruses. Furthermore, there will be described an agglutinin for erythrocytes deriving from several species of animals present in suspensions of normal mouse brain, as well as an inhibitor of agglutination contained in the tissue suspensions and also in normal serum.

*Hemagglutination of Sheep Erythrocytes by Col SK, Col MM, ME, and EMC Viruses.* Dick and Taylor<sup>4</sup> have employed solutions of crystalline bovine plasma albumin (BPA) as a medium for preservation and for dilution

of several viruses, among which were influenza, yellow fever, Lansing and ME. A 0.1 or 0.2% solution of the crystals in buffered saline solution, filtered through a Seitz apparatus generally sufficed for ordinary laboratory purposes, especially for dilution in titration tests.<sup>4</sup> It was also found in this laboratory that for the several viruses employed in the present study BPA was a satisfactory vehicle and the advantages of utilizing a clear, nonagglutinating solution, which also preserved the titer of a virus in tests for hemagglutination, were apparent. Consequently virus-infected mouse brains, (20%) were suspended preferably in 0.1% BPA although saline solution or 10% rabbit serum could also be employed. Such suspensions were used in the fresh state; or if stored, were kept frozen in a mechanical, electrically operated freezer at -20 to -25°C and thawed just before use. Dilutions of virus for hemagglutination were made, however, with buffered saline solution. 0.85% NaCl, 0.05 M phosphate and pH 7.6.

The procedure of the test was as follows: The fresh or thawed viral suspension consisting of 20% brain tissue was centrifuged for clarification at 2,000 rpm for 5 minutes. 0.4 ml of the supernate was added to the first 2 of a series of 10 to 15 tubes. Buffered saline solution in equal amount was introduced into all tubes except the first to make a 2-fold dilution in a final volume of 0.4 ml in the second and successive tubes. To each virus dilution was added 0.4 ml of 0.5% washed sheep RBC suspended in buffered saline solution, thus securing final dilutions of virus of 1:10 to 1:5, 120, or higher, and of the erythrocytes in each tube, 0.25%. Cells were prepared from fresh bleedings and stored in modified Alsever's fluid (ACD):<sup>4\*</sup> as such they could be kept for about 1 month in the ice

\* Lt. Colonel, V.C., U. S. Army.

<sup>1</sup> LaBelle, O., and Horsfall, F. L., Jr., *Proc. Soc. Exp. Biol. and Med.*, 1949, **71**, 713.

<sup>2</sup> Bremer, A., and Mutsaers, W., *C. r. Soc. biol.*, 1948, **142**, 1194.

<sup>3</sup> Hallauer, C., 4th Internat. Cong. Microbiol., July 20-26, 1947, Copenhagen, 1949, p. 257.

<sup>4</sup> Dick, G. W. A., and Taylor, R. M., *J. Immunol.*, 1949, **62**, 311.

<sup>4\*</sup> Rapoport, S., *J. Clin. Invest.*, 1947, **26**, 591.

definitely higher than that of FA virus. With the strains employed in this study, GDVII gave LD<sub>50</sub> titers of the order of  $10^{-8}$  or more while FA gave titers of the order of  $10^{-6}$  or less. On the assumption that the infectivity titer is proportional to the virus concentration, it seemed possible that the failure to demonstrate hemagglutination with FA might be attributable to a relatively low concentration of the agent in infected brain tissue. Because of the numerous similar properties of the 2 viruses<sup>6</sup> and the close antigenic relationship disclosed in the hemagglutination-inhibition experiments described above, it appeared desirable to determine if FA shared with GDVII the capacity to cause hemagglutination of human RBC in the cold.

Attempts to concentrate FA virus, as was feasible with GDVII, by adsorption on human RBC at 4°C and elution in a small volume of diluent at 37°C, were uniformly unsuccessful. In no instance was hemagglutination demonstrable with the eluates despite the use of erythrocytes derived from numerous species. Moreover, the results of infectivity titrations indicated that FA virus was not adsorbed by human RBC under the conditions employed; supernates of virus-RBC mixtures held at 4°C showed no reduction in infectivity titer. It appears, therefore, that despite similarities relative to numerous properties FA virus and GDVII virus do not give similar reactions with human erythrocytes *in vitro*.

**Discussion.** That certain neurotropic viruses possess the capacity to agglutinate in the cold erythrocytes deriving from certain animal species appears evident from the results of this study and that described in the accompanying paper.<sup>5</sup> By means of the hemagglutination reaction with human RBC at 4°C it is possible to estimate *in vitro* the concentration of GDVII virus in a suspension of infected mouse brain. As is the case with other animal viruses which cause hemagglutination, relatively high concentrations are required before positive results are obtained; with GDVII of the order of  $10^4$  mouse infectious doses of virus correspond to one hemagglutinating unit. The available evi-

dence suggests that the infective virus particle is itself responsible for hemagglutination with this agent. By means of the hemagglutination-inhibition technic, also carried out with human RBC at 4°C, the concentration of antibodies in immune serum specifically directed against the virus can be estimated *in vitro*. Evidence obtained in hemagglutination-inhibition experiments indicates that GDVII virus is immunologically closely related to FA virus, but is not related to any other of the numerous agents tested.

Despite numerous attempts with a wide variety of experimental conditions, it was not possible to demonstrate hemagglutination with FA virus. Moreover, with the Lansing, MEF1 and Brunhilde strains of poliomyelitis virus no evidence was obtained indicative of a capacity to combine with erythrocytes. It may be pertinent that neither FA virus nor poliomyelitis virus reaches high titers in infected central nervous system tissue and it is possible that the failure to show hemagglutination with these agents is attributable to insufficient concentration. On the other hand, qualitative factors also may be of critical importance and it seems possible that with erythrocytes from still other species and with different experimental conditions positive results might be obtained.

The dependence of hemagglutination with GDVII virus and of adsorption of the agent by human RBC upon a low temperature, *i.e.* 4°C, appears to be unique. With the exception of the neurotropic viruses discussed in the accompanying paper,<sup>5</sup> other viruses which cause hemagglutination show no such temperature effect.

**Summary.** Suspensions of mouse brain infected with the GDVII strain of mouse encephalomyelitis virus cause agglutination of human Group O RBC at 4°C. Anti-GDVII virus serum inhibits hemagglutination by the agent as also does anti-FA virus serum. GDVII virus is adsorbed by human RBC at 4°C and rapidly elutes from them at 37°C. Three strains of poliomyelitis virus failed to show any evidence of hemagglutination.

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TABLE II.  
Hemagglutination-Inhibition by Antisera: Col SK, Col MM, ME and EMC Viruses and Sheep Cells.

Virus	Antiserum*	Virus. agg. units	Reciprocal of final dilution of serum							
			40	80	160	320	640	1,280	2,560	5,120
Col SK	Col SK	4	0	0	0	0	0	0	0	4
Col SK	NRS†	4	4	4	4	4	4	4	4	4
Col MM	Col MM	4	0	0	0	0	0	1	2	3‡
Col MM	NRS	4	4	4	4	4	4	4	4	4
ME	ME	8	1	0	0	0	0	0	3	4
ME	NRS	8	4	4	4	4	4	4	4	4
EMC	EMC	8	1	0	0	0	0	2	4	4
EMC	NRS	8	4	4	4	4	4	4	4	4
ME	WEE§	8	4	4	4	4	4	4	4	4
EMC	WEE	8	4	4	4	4	4	4	4	4

\* All antisera were prepared by injecting rabbits repeatedly with mouse-brain virus.

† NRS = normal rabbit serum.

‡ The titration continued as follows: 10,240 read 3; 20,480 read 4.

§ Western equine encephalitis antiserum.

period of incubation of antisera and virus was found necessary. The antisera used were prepared by injecting rabbits subcutaneously 3 times at weekly intervals with 1, 2, and 3 respectively, fresh or frozen virus-infected mouse brains. All serum whether immune or normal was inactivated by heating at 56°C for 30 minutes. The virus was kept constant at 4 or 8 units per tube and the serum was diluted 2-fold beginning with 1:10, thus the final dilutions of serum became 1:40 to 1:20,480, since 10 dilutions were usually tested. The test was read after 60 minutes at 5°C. The reading of the hemagglutination inhibition followed standard methods.<sup>5</sup>

Table II demonstrates one of the tests. It will be observed that not only did the anti-

sera inhibit specifically the hemagglutination of sheep cells by the Col SK group of viruses but there was evidence of clear-cut cross-reactions among members of this group of viruses. Table III is presented to demonstrate the results of a test on inhibition of hemagglutination, the titer of antisera and certain cross-reactions among the members of the Col SK group of viruses.

It is concluded therefore that the Col SK, Col MM, ME and EMC viruses agglutinate sheep RBC specifically; they exhibit cross-agglutination inhibition among the individuals of the group and since other neurotropic viruses do not agglutinate sheep cells, as will be shown immediately, these 4 infective agents can be looked upon as having a generic relationship and a common antigenicity. Thus support is given to the findings of Warren and Smadel<sup>6</sup> and of Dick<sup>7</sup> who produced solid evidence from a wholly different approach to the problem of interrelationship of the 4 agents.

Since the hemagglutination is characteristic, it can apparently be utilized for the identification of the viruses of the Col SK group and for measurement of the antibody content of antisera against any of the 4 agents. Finally, since all of the individual members of the group agglutinate sheep RBC in the cold, elute or disperse spontaneously and rapidly at moderate elevations of temperature, and when

TABLE III.  
Tests Showing Hemagglutination-Inhibition Titers of Antisera Against Col SK Group of Viruses and Certain of the Cross-Reactions.

Virus	Antiserum	Agglutination-Inhibition titer
Col SK	Col SK	1:2,560*
	Col MM	1:640
	ME	1:1,280
	EMC	1:1,280
ME	ME	1:2,560
	EMC	1:320
EMC	EMC	1:640
	Col SK	1:640

\* The dilutions represent the highest dilution of antiserum preventing agglutination (Table II).

TABLE I.

Hemagglutination of Sheep Cells by Columbia SK, Columbia MM, Meningo Encephalomyelitis and Encephalomyocarditis Viruses, Held for 120 Min. at 5°C.

Test	Virus or control materials	Reciprocal of final dilution of virus or of normal mouse brain							RBC of types other than sheep, plus virus (10 to 5,120 dils.)
		10	20	40	80	160	320	640 to 5,120	
Test	Col SK	4	4	2	±	0	0	0	0
	Col MM	4	4	2	0	0	0	0	0
	ME	4	3	3	3	2	±	0	0
	EMC	4	4	4	3	2	1	0	0
	West equine	0	0	0	0	0	0	0	0
	East "	0	0	0	0	0	0	0	0
	GDVII	0	0	0	0	0	0	0	+ with human-o cells only
Controls	Normal mouse brain	0	0	0	0	0	0	0	See text
	sheep cells								Cells alone
	Saline soln.		0						0
	BPA		0						0

box; after washing, however, not longer than 5 days. Hamster erythrocytes were prepared from fresh bleedings and were not satisfactorily stored; they were proved useless if kept in ACD for periods longer than 4 days. The tubes were shaken, kept for 60-120 minutes at 5°C and then read. It should be emphasized here that false positives, *i.e.*, nonspecific reactions, easily obscured the results especially since mouse-brain suspensions were employed; the cause of this difficulty will soon be given. Hence tests should include a) a control on the virus suspension, namely, normal mouse brain suspension and b) anti-serum to determine the specificity of hemagglutination. Generally the methods here described and the scale of reading agglutination, except for certain modifications, follow those already described.<sup>1,5</sup> It should be stressed here also that even slight variations in technique sometimes brought about irregular results—a fact which applies also to the standard test.<sup>5</sup>

Table I shows the outcome of one of several similar experiments. The selective agglutination of sheep RBC by Col SK, Col MM, ME and EMC viruses is noted. It is not surprising to find such uniformity of reaction exhibited by the 4 viruses since it has already been found by Warren and Smadel<sup>6</sup> and by Dick<sup>7</sup>

that there is a close relationship among the members of this group as proved by the results of serological, immunological and other biological studies.

With these 4 viruses the hemagglutination was carried out best at 5°C for the reason that spontaneous elution or dispersion of virus from the erythrocytes occurred at room (23°C) or incubator (37°C) temperatures so that at the higher temperature little or no agglutination was visible. This reversibility of the agglutination by means of increasing the temperature could be carried out with the same materials for an indefinite number of times, or as long as sufficient virus survived the process to show its hemagglutinative capacity. In this respect, the present group of viruses behaved as did the GDVII virus.<sup>1</sup>

*Specificity of Hemagglutination.* The next investigation related to the specificity of the hemagglutination for sheep erythrocytes by Col SK, Col MM, ME, and EMC viruses. The procedure followed the established principles<sup>5</sup> of a preliminary titration to determine the hemagglutination titer of the virus to be tested and of selection of a dilution of it which represented not less than 4 and not more than 8 agglutination units. 0.2 ml of this dilution of virus was added to 0.2 ml of anti-sera, then 0.4 cc of the cells—no preliminary

<sup>5</sup> Hirst, G. K., *J. Exp. Med.*, 1942, **75**, 49; Smadel, J. E., in *Viral and Rickettsial Infections of Man*, ed. T. M. Rivers, J. B. Lippincott Co., Philadelphia, 1948, Chap. 3, pp. 77-82.

<sup>6</sup> Warren, J., and Smadel, J. E., *J. Immunol.*, in press.

<sup>7</sup> Dick, G. W. A., *J. Immunol.*, in press.

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Col MM	NRS	4	4	4	4	4	4	4	4	4
ME	ME	8	1	0	0	0	0	0	3	4
ME	NRS	8	4	4	4	4	4	4	4	4
EMC	EMC	8	1	0	0	0	0	2	4	4
EMC	NRS	8	4	4	4	4	4	4	4	4
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\* All antisera were prepared by injecting rabbits repeatedly with mouse-brain virus.

† NRS = normal rabbit serum.

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§ Western equine encephalitis antiserum.

period of incubation of antisera and virus was found necessary. The antisera used were prepared by injecting rabbits subcutaneously 3 times at weekly intervals with 1, 2, and 3 respectively, fresh or frozen virus-infected mouse brains. All serum whether immune or normal was inactivated by heating at 56°C for 30 minutes. The virus was kept constant at 4 or 8 units per tube and the serum was diluted 2-fold beginning with 1:10, thus the final dilutions of serum became 1:40 to 1:20,480, since 10 dilutions were usually tested. The test was read after 60 minutes at 5°C. The reading of the hemagglutination inhibition followed standard methods.<sup>5</sup>

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sera inhibit specifically the hemagglutination of sheep cells by the Col SK group of viruses but there was evidence of clear-cut cross-reactions among members of this group of viruses. Table III is presented to demonstrate the results of a test on inhibition of hemagglutination, the titer of antisera and certain cross-reactions among the members of the Col SK group of viruses.

It is concluded therefore that the Col SK, Col MM, ME and EMC viruses agglutinate sheep RBC specifically; they exhibit cross-agglutination inhibition among the individuals of the group and since other neurotropic viruses do not agglutinate sheep cells, as will be shown immediately, these 4 infective agents can be looked upon as having a generic relationship and a common antigenicity. Thus support is given to the findings of Warren and Smadel<sup>6</sup> and of Dick<sup>7</sup> who produced solid evidence from a wholly different approach to the problem of interrelationship of the 4 agents.

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	EMC	1:1,280
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	EMC	1:320
EMC	EMC	1:640
	Col SK	1:640

\* The dilutions represent the highest dilution of antiserum preventing agglutination (Table II).

freed from the erythrocytes are as active as they were originally, hemagglutination may be useful for purposes of adsorption, without inactivation, of the active agents, just as can be done with the GDVII virus.<sup>1</sup>

*Agglutination Tests with Other Neurotropic Viruses and a Variety of RBC.* A wide variety of erythrocytes other than those of sheep, namely, human O, chick, horse, hamster, dog, cat and guinea pig, were tested for agglutination by the 4 viruses of the Col SK group; the tests failed. In addition, the various erythrocytes just mentioned, including sheep cells, were tested for agglutinability by numerous neurotropic viruses. The viruses employed were:

Eastern equine encephalitis	Theiler (FA strain)
Western equine encephalitis	Theiler (TO strain)
Venezuelan equine encephalitis	poliomyelitis (Lansing strain)
Japanese B encephalitis	poliomyelitis
St. Louis encephalitis	(MEFl strain)
Russian Far East encephalitis	West Nile
vesicular stomatitis (New Jersey strain)	rabies lymphocytic
vesicular stomatitis (Indiana strain)	herpes simplex
	loupung ill

The results of over 100 experiments can be summarized by stating that no specific clumping of any of the types of erythrocytes by any one of the viruses mentioned was detectable. Now and again agglutination was seen but further study revealed it to be nonspecific, most often owing to the physical or particulate condition of the mouse-brain suspension. It was shown that a) normal mouse-brain suspensions produced similar hemagglutination; b) centrifugation to a degree which clarified the suspension but did not sediment the virus from the supernate served to abolish the agglutinative capacity of the supernate, c) antisera failed to inhibit the reaction, d) the reaction was not reversible, e) the aggregations formed did not resemble the "soft", clinging, fine agglomerations as was seen in the hemagglutination by Col SK group of viruses just described. On the contrary, the

aggregates were usually "hard", coarse, irregularly sized and shaped, and sometimes were surrounded by a narrow zone of slight hemolysis.

In view of the fact that GDVII virus exhibits agglutination only of human-O RBC<sup>1</sup> and the Col SK group of viruses only of sheep cells, one may well question the meaning of the negative results obtained with the other neurotropic viruses and the kinds of erythrocytes used in the present investigation. Since agglutination is so selective with respect to cells used, it will not be surprising to find one or another of these viruses yielding positive results with erythrocytes deriving from species not as yet tested.

*Agglutinative Capacity of Normal Mouse Brain Suspensions for Dog, Cat and Guinea Pig RBC.* During the course of the present study, it was noted that suspensions of normal mouse brain agglutinated to a low degree, dog, cat, and guinea pig RBC. The hemagglutination titers were generally 1:10, less often 1:20, rarely 1:40, and of 15 samples in one instance only, 1:320. This reaction was observed after 60 minutes at 5°C, but the maximum titer was reached, however, at room temperature. The reaction was not reversible: there was no visible phenomenon similar to that of spontaneous elution. Moreover, antisera prepared by immunizing rabbits against normal mouse brain had no inhibitory effect on the agglutinative power of the normal mouse brain. The agglutination thus produced revealed, therefore, characteristics unlike those of the viruses of the Col SK group. It is clear, however, that since neurotropic viruses are often employed in the form of infected mouse brain, the occurrence of this nonspecific hemagglutination should be reckoned with in experimental studies.

*Presence of an Agglutinin and an Agglutination-Inhibitor for Hamster Cells in Normal Mouse Brain.* It was also disclosed during the course of the present investigation that there exists in normal mouse brain, and therefore in virus suspensions prepared with infected mouse brain, an agglutinin as well as an agglutination-inhibitor (HI) for hamster erythrocytes, both being present in the same suspensions at the same time.

TABLE IV.

Presence of Hemagglutination-Inhibitor and Agglutination of Hamster RBC by Normal Mouse Brain (NMB) and Virus-Infected Mouse Brain (120 min. at 5°C).

Material used	Dilution of brain tissue or of antiserum*											
	40	80	160	320	640	1,280	2,560	5,120	10,240	20,480	40,960	81,920
NMB	0	0	0	0	0	3	4	4	4	4	4	4
NMB + its anti-serum	0	0	0	0	0	0	2	2	3	3	—	—
NMB + NRS*	0	0	0	0	±	1	4	4	4	4	—	—
West equine virus	0	0	0	1	3	4	4	4	4	4	4	2
West equine virus + its antiserum	0	0	0	1	2	4	4	4	4	—	—	—
NMB heated, 56°C, 10 min.	4	4	4	4	4	4	4	4	4	4	4	4
NMB heated, 56°C, 30 min.	4	4	4	4	4	4	4	4	4	4	2	0

\* NRS = Normal rabbit serum; in an HI test, 8 units of normal or virus-infected mouse brain was used in each tube; reciprocal of final dilution is given.

The agglutinin for hamster cells present in normal and virus-infected mouse brain became evident at ice box or room temperature, maximal after 2 hours' incubation. The agglutinated masses of hamster cells which formed were more minute, delicate and evenly dispersed along the sides of the test tube than were the aggregates formed by the Col SK group of viruses. Specific antisera did not inhibit the hamster-erythrocyte agglutination to any greater extent than did normal serum of the same species. As will be shown later all sera contained a nonspecific inhibitor. It is therefore plain that this agglutinin has characteristics which differ from those shown by the Col SK group of viruses in the presence of sheep cells. The hamster-RBC type agglutination also differs from that observed when normal or infected mouse brain reacted with dog, cat or guinea pig erythrocytes.

The HI exerted its influence in dilutions of 1:40 to 1:160, rarely (3 of 18 titrations) as high as 1:640. After the HI was diluted out hemagglutination was then visible and showed itself in dilutions usually up to 1:80,000 and sometimes higher (Table IV). The HI was active both at ice-box and room temperatures and the maximum titers were noted after 2 hours' incubation. The inhibitor was ther-

molabile and could be inactivated by heating mouse-brain suspensions at 56°C for 10 to 30 minutes at which temperature the agglutinin was not affected (Table IV). Sodium citrate 2.5% did not neutralize its effect as it does in the case of PVM and other viruses.<sup>8</sup> It could not be sedimented out of a suspension of mouse brain at 3,000 rpm for 10 minutes and rabbit antisera prepared by repeated injection of normal or virus-infected mouse brain failed to reduce its titer of activity.

*Nonspecific HI in Certain Normal Serum and Antisera.* Antisera prepared in rabbits, guinea pigs, or monkeys, and the respective normal sera of these species possessed the capacity to inhibit hamster-cell agglutination by normal or virus-infected mouse-brain suspensions. Thus the nonspecific inhibition could be demonstrated in dilutions of sera up to 1:1,280 (Table IV). When normal guinea pig or rabbit serum was heated at 65°C for 30 minutes the contained nonspecific inhibitor was not thereby inactivated. It has been previously reported<sup>8,9</sup> that such treatment of serum can inactivate the inherent nonspecific inhibitor of hemagglutination by other viruses.

<sup>8</sup> Ginsberg, H. S., personal communication.

<sup>9</sup> Ginsberg, H. S., and Horsfall, F. L., Jr., *J. Exp. Med.*, 1949, **89**, 37.



*Summary of Nonspecific Agglutinins and Inhibitors.* In sharp contrast to the clearly defined, specific agglutination of sheep red cells by the Col SK group of viruses on one side, and the failure of many other neurotropic viruses to show hemagglutination on the other, is the existence of the following non-specific elements:

a) An agglutinin for dog, cat and guinea pig erythrocytes is contained in normal, or virus-infected mouse brain.

b) An agglutinin for hamster red cells is present in normal or virus-infected mouse brain.

c) A thermolabile inhibitor of agglutination of hamster RBC exists concomitantly with the agglutinin for the hamster cells just mentioned, in normal or virus-infected mouse brain.

d) A thermostable inhibitor is present in normal monkey, rabbit and guinea pig serum, consequently in antisera as well, which prevents agglutination of hamster cells.

The nonspecific reactions just described require further study for the identification of the agglutinins and the inhibitors present in mouse brain suspensions and in normal serum. For the moment, the use of erythrocytes deriving from hamsters, dogs, cats and guinea pigs for agglutination by neurotropic viruses in the form of mouse-brain suspensions would appear to require caution. Since all the standard hemagglutination tests, now routine in laboratory practice, require careful control of the variable employed, similar precautions are needed as well for the Col SK, Col MM, ME, and EMC viruses.

*Conclusions.* Evidence has been brought forward to indicate that Columbia SK, Co-

lumbia MM, Mengo encephalomyelitis and encephalomyocarditis viruses agglutinate sheep red cells. It is therefore possible to identify these viruses by means of hemagglutination and to measure the antibody content of antisera. Since the viruses show characteristic spontaneous elution or dispersion from the erythrocytes, the method can be used for purposes of selective adsorption of the viruses without loss of their hemagglutinative activity. Moreover, the uniformity of the hemagglutination reaction shown by the 4 viruses and the cross-inhibition that exists among them supports the findings of Warren and Smadel<sup>6</sup> and of Dick<sup>7</sup> that these viruses are similar in many respects and are of the same group.

Seventeen other neurotropic viruses were tested for their capacity to agglutinate characteristically erythrocytes deriving from sheep, man (group O), chicken, horse, hamster, dog, cat and guinea pig; these tests failed.

Another phenomenon that was observed is the nonspecific agglutination of dog, cat and guinea pig erythrocytes by normal or virus-infected mouse brains. With respect to agglutination of hamster cells, an inhibitor of agglutination is present not only in suspensions of normal mouse brain but also in normal serum and antiserum against the neurotropic viruses.

The fact that neurotropic viruses are often used in the form of mouse brain suspensions renders it important therefore for investigators to use proper controls for the variable of the test and, in addition, to identify positive reactions by specific means.

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17310. Veriloid, a New Hypotensive Extract of *Veratrum Viride*.\*

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The promising results of Freis *et al.*<sup>1,2</sup> in therapy of patients with essential hypertension suggested that a hypotensive extract of constant composition from *Veratrum viride* would be highly useful. Accordingly a series of fractionations was carried out.<sup>3</sup> An assay routine based on hypotensive properties in normal dogs under pentobarbital anesthesia was applied. Accessory screening technics included emetic and bradycardic potency estimations. After screening some 75 fractions, a highly potent, reproducible, stable and uniform extract was selected for further study. This material has been given the proprietary name "Veriloid."

While the physical and chemical properties of "Veriloid" will be treated *in extenso* elsewhere a brief outline is presented in Table I. Evidence exists that "Veriloid" is a mixture and not a single alkaloid. None of the previously described potent alkaloids of *Veratrum viride* has been obtained by fractionation of "Veriloid." (See Krayer<sup>3</sup> for comprehensive review of literature). The relatively impotent rubijervine and isorubijervine have been identified as present in the mixture to the extent of 25%. For clinical use further purification at present was not warranted for logistic reasons. A series of 12 separate fractionations from crude root has shown inappreciable variations in product by both chemical and biological tests.

Biological effects of "Veriloid" are outlined

\* Supported in part by a grant from the Coe Chemical Co., Los Angeles, Calif.

† Product Development Department, Coe Chemical Co.

<sup>1</sup> Fries, E. D., and Stanton, J. R., *Am. Heart J.*, 1948, **36**, 723.

<sup>2</sup> Fries, E. D., Stanton, J. R., Culbertson, J. W., Litter, J., Halperin, M. H., and Wilkins, R. W., *J. Clin. Inv.*, 1949, **28**, 353.

<sup>3</sup> In the laboratories at Los Angeles.

<sup>3</sup> Krayer, O., and Acheson, G. H., *Physiol. Rev.*, 1946, **26**, 353.

in Table II. Full data will be published separately.

The data illustrate a high therapeutic ratio for hypotensive action. With increasing intravenous dosage in normotensive animals, the degree of hypotension increased only up to an average maximum fall of 40% of the pre-existing mean arterial pressure. As dosage was raised the duration of the reduced mean pressure increased. Usual duration at fully effective intravenous dosage was 30 to 90 minutes. The return to previous pressure was gradual. Preliminary data indicated that the role of the bradycardia in the hypotension was a secondary one. Hypotension still was elicited after vagotomy; bradycardia was not. The hemodynamic rearrangement seen during this hypotension would best fit a hypothesis that "Veriloid" caused dilatation of arterioles in skeletal muscle, splanchnic region and skin accompanied by constriction of venous vascular beds. Evidence was elicited that blood flow was not reduced with the hypotension. Systolic and diastolic blood pressures were both decreased and nearly equally so. Anesthesia did not alter the minimal hypotensive dose. Hypotension resulting from "Veriloid" was corrected by pressor amines such as epinephrine, phenylephrine and methoxamine.

Minimal bradycardic action from intravenous administration had a higher minimal effective dose, a longer latency and a shorter duration than did the hypotensive action. Pentobarbital anesthesia reduced the amount of drug required to produce bradycardia. As the dose was raised degree and duration of bradycardia increased. Lengthening of P-R interval, partial heart block, and transient A-V nodal rhythm were seen at 20 to 50 times the minimal dose. As the dose was raised still further ventricular tachycardia, venous and arterial hypertension resulted. A coarse ventricular fibrillation was observed occasionally at 200 to 500 times the minimal dose. Very high intravenous dosage in the unanesthetized

TABLE I.  
Physical and Chemical Properties of "Veriloid."

Appearance: Pale yellow amorphous powder.

Melting Point: Sinters at 102°-105°C.  
Melts at 148°-155°C.

Optical Rotation:  $\alpha_D^{26}$  in EtOH =  $-17.3^\circ\text{C} = 0.5 \text{ g}/100 \text{ ml}$ .

Ultra Violet Absorption Curve: Peak at 2500 Å  $\ln E = 3.30$  (at 0.00008 g/ml).

Solubility: In water—very slightly soluble.

In dilute acid—soluble.

In benzene, alcohol, chloroform, propylene glycol, acetone—soluble.

Spot test reaction with c.  $\text{H}_2\text{SO}_4$ —Dark orange going to reddish orange\* to brown in 24 hours.

Nitrogen Content—2.9%.

TABLE II.  
Dosage of "Veriloid" in mg/kg Required to Produce Physiological Alterations in Dogs.

Effects	Anesthetized (Pentobarbital sodium) Intravenous	Unanesthetized	
		Intravenous	Oral
Hypotension (reduction of at least 10 mm Hg)	ED50 $0.0021 \pm 0.0001^*$ (200 trials)	ED50 ca 0.002 (26 trials)	Irregular
Bradycardia	ED50 $0.0021 \pm 0.0003$ (75 trials)	ED50 $0.0055 \pm 0.0004$ (75 trials)	Not obtained at 0.05 (20 trials)
Emesis—Fasted	Varies with depth of depression. Rare		ED50 $0.0136 \pm 0.0017$ (50 trials)
Not fasted		ED50 $0.0197 \pm 0.001$ (50 trials)	ED50 ca 0.025 (30 trials)
Apnea (30 to 90 sec. duration)	Irregular but never below 0.02 (200 trials)	ED50 ca 0.1 (15 trials)	Never obtained (Tried to 5.0)
Hyperirritability (15 to 20 sec. duration)	Never obtained	Above 0.01 (45 trials)	Never obtained
Displacement of cardiac pacemaker	Approximately 0.1 (20 trials)	Never below 0.05. Occa- sionally at 0.1. Common at 0.2 to 0.5	Seen at 1.0
Hypertension	ED50 ca 0.05 (30 trials)		
Lethal	Varies 0.05 to 0.4 (30 trials)	LD50 ca 0.5 (6 trials)	Never obtained 5.0 tolerated (5 trials)

\* Standard error (See Miller and Tainter, *PROC. SOC. EXP. BIOL. AND MED.*, 1944, **37**, 261).

animal produced similar effects except that fibrillation has never been observed. Transient A-V nodal rhythm has also been obtained after oral administration of 1 mg/kg in the dog.

Tachyphylaxis to the bradycardic and hypotensive actions has not been seen. Similarly, tolerance did not develop in 2 dogs following the intramuscular administration of 5 times the minimal effective dose twice a day

for 5 weeks.

Like other *Veratrum* derivatives "Veriloid" is a potent emetic by any route. Oral dosage required to produce vomiting was at least doubled by the presence of food in the stomach. Thus reduction of clinical side effects would be expected to result from administration during meals.

Oral administration in dosage up to 0.075 mg/kg to the unanesthetized dog has only irregularly resulted in hypotension. The same dose did cause a fall of mean arterial blood pressure when placed in the upper jejunum of animals anesthetized with pentobarbital. Clinically, the effective single oral dose has been found to vary from 0.03 to 0.08 mg/kg in hypertensive patients.<sup>4</sup>

Comparison of the effects of intravenous and intestinal administration in the anesthetized dog showed that the drug was more effective in producing fall of blood pressure by the intravenous route. In order to delineate the effect of slow absorption prolonged infusions were made. Slow intravenous ad-

ministration became ineffective in the anesthetized dog below  $\frac{1}{4}$   $\gamma$ /kg/min. Effective doses by this method produced graded maintained hypotension. Comparison of the effects after splenic and femoral intravenous administration has not demonstrated inactivation of "Veriloid" by the liver.

Limited clinical trial of "Veriloid" has been accomplished by Freis and Wilkins. This will be reported separately. Certain individuals required as little as 2 mg or as much as 6 mg for single oral dose to produce blood pressure fall. Latency for full hypotension to be established was 2 hours.

**Summary.** A stable, reproducible and highly potent extract of *Veratrum viride* has been described. The name "Veriloid" has been given to this preparation. Methods for biological control of potency have been developed. In normal dogs the extract was hypotensive, bradycardic and emetic. Pharmacologic results suggest that the hypotensive action of the extract deserves trial in the treatment of human hypertension.

<sup>4</sup> Wilkins, R. W., and Freis, E. D., personal communication.

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### 17311. A Comparison of Desoxyribonucleic Acid Content in Certain Nuclei of Normal Liver and Liver Tumors.

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Direct chemical analyses of actively growing tissues, particularly tumors, for desoxyribonucleic acid have been reported by several investigators with, however, scant agreement. The percentage of desoxyribonucleic acid in nuclear material was found by Dounce<sup>1</sup> to be the same for Walker carcinosarcoma 256 and normal liver; in hepatoma 31 the percentage of desoxyribonucleic acid was lower than the normal. Brues, Tracy and

Cohn<sup>2</sup> observed that the phosphorus and nitrogen content of hepatoma 31 and normal liver was the same per unit weight. On a similar basis, Davidson and Waymouth<sup>3</sup> and Schneider<sup>4</sup> recorded values for desoxyribonucleic acid that were higher in hepatomas than in normal liver.

Using the light absorption of fields of Feulgen stained nuclei, Stowell<sup>5</sup> also found

\* Fellow of the Jane Coffin Childs Memorial Fund for Medical Research. This investigation was aided by grants from the Jane Coffin Childs Memorial Fund for Medical Research.

<sup>1</sup> Dounce, A. L., *J. Biol. Chem.*, 1943, 151, 235.

<sup>2</sup> Brues, A. M., Tracy, M. M., and Cohn, W. E., *J. Biol. Chem.*, 1944, 155, 619.

<sup>3</sup> Davidson, J. N., and Waymouth, C., *Biochem. J.*, 1944, 38, 379.

<sup>4</sup> Schneider, W., *Cancer Res.*, 1945, 5, 717.

<sup>5</sup> Stowell, R. E., *Cancer Res.*, 1946, 6, 426.

TABLE I.  
Relative Amount of Desoxyribonucleic Acid in the Nuclei of Normal Liver and of Liver Tumors.

Cell type	No. nuclei	(E) extinction coefficient	(A) nuclear area	(E) × (A) relative amount of DNA
Slide 1				
Normal hepatic	10	.161-.187 (.177)	23	4.09 ± .14
Hepatoma	10	.237-.260 (.247)	16.5	4.09 ± .12
Slide 2				
Normal hepatic	5	.208-.244 (.227)	23	5.22 ± .20
Cholangioma	10	.222-.265 (.225)	23	5.19 ± .23

higher values for the desoxyribonucleic acid in various tumors; here the results represented the average amount of desoxyribonucleic acid in many nuclei. The actual amount of desoxyribonucleic acid per single nucleus was not determined, nor was there any assay of the partition of the desoxyribonucleic acid into nuclear classes bearing different numbers of chromosomes.

Recent chemical determinations of the desoxyribonucleic acid in isolated nuclei by Boivin *et al.*,<sup>6</sup> Vendrely and Vendrely,<sup>7</sup> and Mirsky and Ris,<sup>8</sup> have demonstrated a remarkable species constancy in the desoxyribonucleic acid content of nuclei with the same number of chromosomes. In view of these findings, an attempt was made to establish the validity of this relationship for the presumably abnormal nuclei of tumors.

In the present study the amount of desoxyribonucleic acid in single nuclei of the same size has been compared in normal and tumor tissue utilizing the intensity of the Feulgen reaction. Precise data on the desoxyribonucleic acid content of single nuclei obtained by Mirsky and Ris<sup>8</sup> made it possible to use the Feulgen reaction as a measure of the relative amount of desoxyribonucleic acid per nucleus (Ris and Mirsky<sup>9</sup>).

The normal and tumor tissues studied were taken from the white rat. Liver tumors, hepatomas and cholangiomas, were induced by a diet of brown rice and carrot containing

0.06% p-dimethylaminoazobenzene as originally reported by Kinoshita,<sup>10</sup> and were histologically identified according to the criteria described by Opie.<sup>11</sup> Tissue blocks were fixed in 10% formalin and cut at 10  $\mu$  thickness.

As elaborated elsewhere (Pollister and Ris;<sup>12</sup> Ris and Mirsky,<sup>9</sup>) the apparatus used in the measurements consisted of a Spencer monocular microscope with a mercury vapor arc as light source and a phototube and galvanometer stationed above a variable diaphragm in the image plane. The light absorption of individual nuclei stained by the Feulgen reaction was measured at a wave length of 546 m $\mu$ .

In Table I comparative measurements are shown of the smallest spherical nuclei in Feulgen stained paraffin sections of normal and tumor tissue mounted together on the same slide. The relative value for desoxyribonucleic acid per nucleus in the normal cell and in the hepatoma cell appears the same, as in slide 1. Similarly, slide 2 indicates the same desoxyribonucleic acid content per nucleus for the normal hepatic cell and the cholangioma cell.

Since the method is limited to the measurement of spherical nuclei, it was not possible to compare irregular bile duct nuclei with cholangioma nuclei directly, or to measure large nuclei of bizarre shape. Certain variations may be found in the values if the Feulgen technic varies slightly, as in Table I. Only sections on the same slide, therefore, are comparable.

<sup>6</sup> Boivin, A., Vendrely, R., and Vendrely, C., *Compt. Rend. Acad. Sci.*, 1948, **226**, 1061.

<sup>7</sup> Vendrely, R., and Vendrely, C., *Experientia*, 1948, **4**, 434.

<sup>8</sup> Mirsky, A. E., and Ris, H., *Nature*, 1949, **163**, 666.

<sup>9</sup> Ris, H., and Mirsky, A. E., unpublished data.

<sup>10</sup> Kinoshita, R., *Trans. Jap. Path. Soc.*, 1937, **27**, 665.

<sup>11</sup> Opie, E. L., *J. Exp. Med.*, 1944, **80**, 231.

<sup>12</sup> Pollister, A. W., and Ris, H., *Cold Spring Harbor Symposia Quant. Biol.*, XII, 1947.

The results indicate that in the smallest spherical nuclei of liver tumors the amount of desoxyribonucleic acid is the same as in normal hepatic nuclei of similar size. Comparable results were independently obtained by Leuchtenberger,<sup>13</sup> who measured nuclei in transplanted mouse sarcoma. Considering the abnormal chromosome arrangements which are known to exist in neoplasms, (Boveri<sup>14</sup>) the presence in these tumors of nuclei with more or less nucleic acid cannot be excluded.

<sup>13</sup> Leuchtenberger, C., personal communication.

<sup>14</sup> Boveri, T., *The Origin of Malignant Tumors*, Williams and Wilkins, Baltimore, 1929.

The conflicting results of previous investigations may be attributed in part to the analyses of tissue samples containing different numbers and different sizes of nuclei.

*Summary.* The amount of desoxyribonucleic acid in single spherical nuclei of normal rat liver was compared with that of similar nuclei in tumor tissue by microphotometric determination of the intensity of the Feulgen reaction. It was found that the amount of desoxyribonucleic acid contained in nuclei of similar size is the same in hepatoma and cholangioma as in the normal liver.

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### 17312. Excretion of Radiocalcium by Normal Rats.\*

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The objectives of the present experiments were to determine the effect of various amounts of labeled calcium on the relative amounts of radiocalcium<sup>†</sup> excreted in the urine and feces, and to determine the distribution of radiocalcium in the tissues and contents of the gastrointestinal tract during the first few hours after the administration of a dose of labeled calcium.

*Materials and methods.* Young adult rats of the Sprague-Dawley strain were used. Immediately after the subcutaneous injection of a dose of labeled<sup>§</sup> calcium each animal was placed in a separate wire metabolism cage over a urine-feces separator.<sup>1</sup> The animals

shown in Table II were supplied with water only, but each of the other animals had access to both water and the stock diet, Purina Laboratory Chow, during the experimental period.

At the end of the experimental period the animals were dispatched by a blow on the head. In those instances in which the segments of the gastrointestinal tract and their contents were to be separately assayed for radioactivity, especial care was taken to remove the entire contents. The collected urine and cage washings, the feces, and the other samples were dried, and then dry ashed in an electric muffle at 550°C. In each instance the ash was dissolved in dilute hydrochloric acid and suitable aliquots transferred to small aluminum pans and dried under an infra red lamp. Dry sample weights were kept small (0.5 to 1.5 mg/cm<sup>2</sup> on sample pan surface) and comparable to the standards in order to minimize the error due to self-absorption of radioactivity. Suitably prepared standards and the samples were then assayed for radio-

\* This investigation was greatly aided by a grant from the Endocrine Committee of the National Research Council. A part of the equipment used in this work was purchased with funds supplied by the Ella Sachs Plotz Foundation.

<sup>†</sup> Present address: Radioisotope Unit, Veterans Administration Hospital, Hines, Ill.

<sup>‡</sup> The radiocalcium (Ca<sup>45</sup>) used in these experiments was allotted by the United States Atomic Energy Commission, and supplied by the Monsanto Chemical Company.

<sup>§</sup> 3 to 10 microcuries of Ca<sup>45</sup>.

<sup>1</sup> Gross, I., and Connell, S. V. B., *J. Physiol.*, 1923, 57, ix.

TABLE I.  
Excretion of Radiocalcium after Injection of Various Amounts of Labeled Calcium.

No. of rats	Body wt., g	Dose of labeled Ca, mg	% of dose excreted	
			Urine	Feces
8	208 $\pm$ 12*	0.07	0.59 $\pm$ 0.25	12.60 $\pm$ 5.75
4	293 $\pm$ 30	0.21	0.61 $\pm$ 0.42	9.56 $\pm$ 1.13
3	224 $\pm$ 40	0.41	0.93 $\pm$ 0.65	11.36 $\pm$ 1.25
3	213 $\pm$ 12	2.50	5.90 $\pm$ 1.75	15.17 $\pm$ 1.14
3	190 $\pm$ 8	4.90	2.95 $\pm$ 0.94	12.46 $\pm$ 1.10
6	211 $\pm$ 30	7.00	2.49 $\pm$ 1.55	9.41 $\pm$ 5.40
10	233 $\pm$ 35	14.00	6.05 $\pm$ 2.15	7.74 $\pm$ 4.50

\* Standard deviation of the mean.

activity by using a scale-of-sixteen Geiger-Muller counter, equipped with a thin mica window (1.8 mg/cm<sup>2</sup>) tube.

**Results and comments.** The data summarized in Table I show that under the conditions of our experiments subcutaneously administered radiocalcium was excreted mainly in the feces. Variations in the relative amounts of radiocalcium excreted in the urine and feces were large for each dose of labeled calcium injected, but the results indicate clearly that the amount of calcium in the dose was an important factor in determining the relative amounts of radiocalcium excreted in the urine and feces. As would be expected, retention of the radiocalcium varied widely and probably reflects differences in the rate of absorption of the labeled calcium, or differences in the calcium requirement of the animals.

Fig. 1 shows graphically the excretion of radiocalcium by 2 groups of rats during the 9 days following administration. The animals of Group 1, each of which received an injection of 7 mg of labeled calcium at the beginning of the experimental period and a second injection of 7 mg at the end of the first hour, excreted 5.2% of the administered radiocalcium in the urine and 4% in the feces during the first 24 hours. Thereafter the amounts of the radiocalcium which appeared daily in the feces exceeded the amounts excreted in the urine. Summation of the fractions of the administered radiocalcium found in the urine and feces during the 9-day experimental period reveals that approximately 7.27% of the dose was excreted in the urine and 15.9% in the feces.

The results obtained for the animals of Group 2, Fig. 1, show that when the quantity of labeled calcium injected was only 0.07 mg the average amount of the administered radiocalcium excreted in the urine during the first 24 hours was only 0.66%, while the average amount excreted in the feces was 8.49%. In the case of this group of animals approximately 2.10% of the dose of labeled calcium was excreted in the urine and 22.75% in the feces during the 9-day experimental period. From these experiments (Fig. 1) it is apparent that the gastrointestinal tract was the principal route by which the labeled calcium was excreted. Furthermore, it appears that only when a plethora of the labeled calcium was being eliminated during the first 24 hours did the kidneys respond by excreting a large fraction of the administered radiocalcium.

It is generally agreed that a considerable amount of excreted calcium enters the lumen

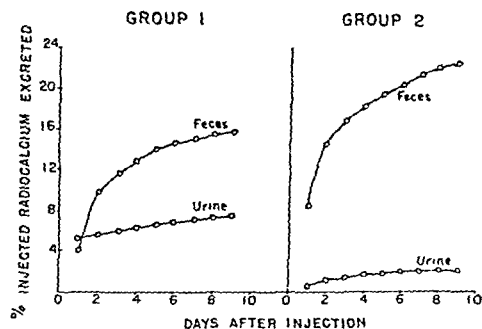


FIG. 1.

Group 1 consisted of 4 rats (avg wt. 265 g), each of which received two subcutaneous doses of 7 mg of labeled calcium within the first hour of the experimental period. Group 2 consisted of 4 rats (avg wt. 220 g), each of which received a single subcutaneous dose of 0.07 mg of labeled calcium at the beginning of the experimental period.

TABLE II.  
Distribution of Radiocalcium in Tissues and Contents of Gastrointestinal Tract.

Rat No.	Body wt., g	Hr after dose of 7 mg labeled Ca	% of dose recovered from tissues				% of dose recovered from contents			
			Stomach	Small intestine	Cecum	Colon	Stomach	Small intestine	Cecum	Colon
1	205	1	0.14	0.40	0.01	0.08	0.23	1.79	0.29	0.12
2	190	1	0.11	0.60	0.02	0.11	0.12	0.91	0.10	0.06
3	204	1	0.10	0.69	0.03	0.07	0.15	0.92	0.14	0.16
4	185	1	0.20	0.40	0.02	0.07	0.11	1.10	0.12	0.07
Avg	196		0.14	0.52	0.02	0.08	0.15	1.18	0.16	0.10
5	178	3	0.17	0.49	0.03	0.14	0.09	1.24	0.57	0.35
6	196	3	0.12	0.63	0.04	0.12	0.05	1.23	0.17	0.13
Avg	187		0.14	0.55	0.03	0.13	0.07	1.23	0.37	0.24
7	190	6	0.11	0.44	0.03	0.13	0.05	0.41	1.71	0.17
8	187	6	0.12	0.37	0.01	0.10	0.08	0.48	1.17	1.57
9	186	6	0.14	0.71	0.04	0.28	0.03	0.41	1.41	0.14
Avg	188		0.12	0.57	0.03	0.17	0.05	0.43	1.43	0.63

of the intestine as a constituent of the intestinal secretions<sup>2</sup> and the bile,<sup>3</sup> and that this calcium, supplemented by a fraction of the dietary calcium is reabsorbed. Evidence of an active excretion of calcium through the wall of the small intestine,<sup>4</sup> or the colon,<sup>5-7</sup> however, has not been equally acceptable.

In our experiments (Table II) the employment of radiocalcium has made possible the orientation of various portions of the gastrointestinal tract with respect to the excretion of calcium. The distribution patterns of radiocalcium in the tissues and contents of the gastrointestinal tracts of animals, which were sacrificed at 1, 3, and 6-hour intervals after the subcutaneous injection of 7 mg of labeled calcium, are shown in Table II. Of particular interest was the relatively large fraction of the excreted radiocalcium found in the contents of the small intestine and the smaller fractions recovered from the contents of the cecum and colon at the 1-hour interval. In Rats 1 and 2 the small intestine was severed at

the midpoint and the tissues and contents of the two halves separately assayed for radioactivity. The tissues of the upper and the lower half of the small intestine of Rat 1 contained 0.24 and 0.16% of the administered radiocalcium, respectively, whereas the contents of the upper and lower half contained 0.43 and 1.36%, respectively. In the case of Rat 2 the tissues of upper and lower half of the small intestine contained 0.28 and 0.32% of the administered radiocalcium, respectively, and the contents 0.13 and 0.78% respectively.

Unfortunately separate assays were not made on the tissues and contents of equal parts of the small intestine of Rat 5 or 6. However, such assays were made on Rats 7, 8, and 9. In these animals approximately the same amounts of radio-activity were found in the tissues of the upper and lower half of the small intestine, but in 2 instances the amounts of radiocalcium in the contents of the 2 segments differed appreciably. In Rat 7 the contents of the upper and lower half of the small intestine contained 0.12 and 0.29% of the injected radiocalcium, respectively, and in the case of Rat 9 the fractions of the administered radiocalcium recovered in the 2 segments were 0.09 and 0.32%, respectively. The contents of the upper and lower half of the small intestine of Rat 8 contained 0.20

<sup>2</sup> Logan, M. A., *Physiol. Rev.*, 1940, **20**, 522.

<sup>3</sup> Greenberg, D. M., and Troescher, F. M., *Proc. Soc. Exp. Biol. and Med.*, 1942, **49**, 488.

<sup>4</sup> Walsh, E. L., and Ivy, A. C., *Proc. Soc. Exp. Biol. and Med.*, 1928, **25**, 839.

<sup>5</sup> Bergeim, O. J., *J. Biol. Chem.*, 1926, **70**, 51.

<sup>6</sup> Cowell, S. J., *Biochem. J.*, 1937, **31**, 848.

<sup>7</sup> Kosman, A. J., and Freeman, S., *Fed. Proc.*, 1943, **2**, 16.



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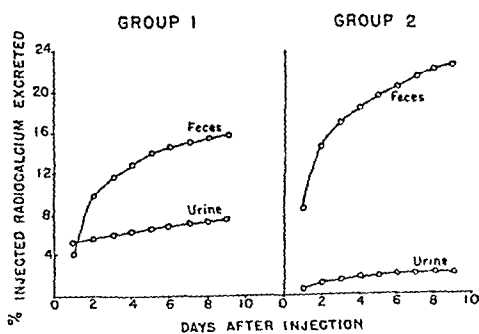


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PREPARED BY DR. EMIL BAUMANN

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and 0.28% of the injected radiocalcium, respectively.

It is very doubtful that the major portion of the excreted radiocalcium found in the lower half of the small intestine could have resulted from the failure of the upper half to reabsorb secreted calcium. Duckworth and Godden<sup>8</sup> have observed in the rat that the net absorption of ingested calcium is not affected by a diet containing 30% of fiber. Since such a diet should cause a great increase in the intestinal secretions, it would appear that the efficiency of reabsorption of secreted calcium is very high in the rat. Therefore, we are inclined to regard that fraction of the excreted radiocalcium found in the lower half of the small intestine as indicative of an active excretion of calcium through the wall of the intestine. In this connection, the extreme rapidity with which the fecal excretion of calcium has been shown to occur is of great interest. Norris and Kisielski<sup>9</sup> recovered 5 to 8% of a dose of labeled calcium from the contents of the intestines of rats one minute after intravenous administration.

The presence of relatively small fractions of excreted radiocalcium in the contents of the cecum and colon at the 1 hour interval (Table II), and the larger amounts in the contents of these segments at later intervals, suggest that the bulk of the excreted radio-

calcium entered the small intestine at points above the ileocecal valve. This observation is in accord with evidence that has been advanced<sup>10</sup> that there is little or no active excretion of calcium into the large intestine.

*Summary.* 1. The excretion of subcutaneous-ly administered radiocalcium has been studied in normal rats.

2. The amount of labeled calcium injected appeared to be an important factor in determining the relative amounts of radiocalcium excreted in the urine and feces. When the quantity of labeled calcium injected was increased from 0.07 mg to 14 mg the fraction of the excreted radiocalcium which appeared in the urine in the first 24 hours increased from a relatively small value to a value approaching or slightly exceeding the amount of radiocalcium excreted in the feces. During a 9-day experimental period the amount of radiocalcium excreted in the feces, when either 0.07 or 0.14 mg of labeled calcium was administered, greatly exceeded the amount excreted in the urine.

3. The distribution of radiocalcium in the tissues and contents of the stomach, small intestine, cecum, and colon was determined at 1, 3, and 6-hour intervals after the administration of a dose of labeled calcium. The results of these experiments indicate that there is little or no active excretion of calcium through the wall of the large intestine.

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PREPARED BY DR. EMIL BAUMANN

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